

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF PLANT
GROWTH PROMOTING PUTATIVE ENDOPHYTIC BACTERIUM ISOLATED
FROM *IN VITRO* CULTURES OF *PHOTINIA X FRASERI* DRESS.**

IRMAK ŞAH
A THESIS SUBMITTED FOR THE DEGREE OF
DOCTORATE OF PHYLOSOPHY
DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

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2017

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***IN VİTRO* KÜLTÜRDEN İZOLE EDİLEN ALEV**
AĞACIYLA İLİŞKİLİ BİTKİ BÜYÜMESİNİ TEŞVİK
EDEN ENDOFİTİK BAKTERİNİN BİYOKİMYASAL VE
MOLEKÜLER KARAKTERİZASYONU

IRMAK ŞAH
DOKTORA TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

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SUMMARY

Fraser photinia “Red Robin” (*Photinia X fraseri*) is an evergreen, ornamental hybrid shrub and has a commercial significance. The microshoots of fraser photinia are used routinely for the optimization of long-term storage techniques. A microorganism is considered endophytic if it is obtained by extraction from surface sterilized plant material or plant. The most basic property that distinguishes endophytic organisms from pathogens is that they do not have any harmful effect on the host. In the Gebze Technical University (GTU), Plant Biotechnology Laboratory, the presence of beneficial bacterium in plant tissue culture was detected during the micropropagation of photinia. In the scope of the thesis, microbiological, biochemical and molecular analyzes have been carried out for identification and detection of positive effects of this endophytic bacterium isolated from *in vitro* cultures of fraser photinia microshoots. It has been determined by standard biochemical tests that this bacterium is capable of producing GA₃ and indole acetic acid, is sensitive to streptomycin, tetracycline, nalidixic acid, polymyxin B, spectinomycin and erythromycin antibiotics, is spore-free, Gram negative, rod-shaped, and also has the ability to fix nitrogen. Molecular analysis revealed that the auxin, cytokinin, nitrogen fixing, and virulence genes in the bacteria were present in different sizes according to the control microorganisms. It has been shown that fraser photinia microshoots can be stored at 25 °C for up to 16 months without subculturing or transferring a fresh medium when inoculated with putative endophytic bacterial culture. With the presence of this bacterium subculture period was extended which means that the plants can protect their vitality for a long time without adding any external additives to the medium and reduce labour, material and energy loss. In this context, this thesis is important in terms of utilization of endophytic bacterium-plant collaboration.

Key Words: Fraser Photinia, Endophytic Bacteria.

ÖZET

Alev ağacı (*Photinia X fraseri* Dress.) her mevsim yeşil, ticari öneme sahip odunsu bir süs bitkisidir. Bir mikroorganizma, yüzey sterilasyonu yapılmış bitki materyalinden ya da bitki içinden ekstraksiyonla kazanılıyorsa endofitik olarak değerlendirilmekte olup; endofitik organizmaları patojenlerden ayıran en temel özellik konakçı üzerinde herhangi zararlı bir etki göstermemeleridir. Gebze Teknik Üniversitesi (GTU), Bitki Biyoteknolojisi Laboratuvarında, alev ağacı bitki doku kültürü çalışmalarının dördüncü yılında, bitki büyümesini teşvik eden endofitik olabilecek bir bakterinin varlığı saptandı. Tez kapsamında *in vitro* koşullarda alev ağacı bitkisinden izole edilen endofitik bakterinin tanımlanması ile gösterdiği olumlu etkinin kaynağının açığa çıkarılmasına yönelik mikrobiyolojik, biyokimyasal ve moleküler analizler yapılmıştır. Yapılan standart biyokimyasal testler ile bu bakterinin sporsuz, Gram negatif, çubuk şekilli olduğu, streptomisin, tetrasiklin, nalidiksik asit, polimiksin B, spektinomisin ve eritromisin antibiyotiklerine hassas olduğu, GA₃ ve indol asetik asit ürettiği ve ayrıca nitrojeni fikse etme yeteneğine sahip olduğu belirlenmiştir. Yapılan moleküler analizlerle bakteride oksin, sitokinin ve virülens genlerinin control mikroorganizmalara göre farklı büyüklükte olsalar da bulunduğu saptanmıştır. Alev ağacı mikrogövdelerinin saf olarak izole edilen bakteri kültürü ile inoküle edildiğinde periyodik altkültürlemeye gereksinim duymaksızın 25 °C'ta 16 aya kadar saklanabildikleri görülmüştür. Sonuçların değerlendirilmesi ile, bitki doku kültüründe yararlı bakteri varlığının alt kültürleme süresini uzattığı ve bu sayede bitkiciklerin besi ortamına dışarıdan herhangi bir katkı yapılmaksızın uzun süre canlılıklarını koruyabildikleri belirlenmiştir. Dolayısıyla, doku kültürü ortamında yararlı bakteri varlığı; alt kültürleme sıklığını azaltmakta, iş gücü, materyal ve enerji kaybını önlemektedir. Bu bağlamda, bu tez endofitik bakteri-bitki iş birliğinden yararlanılması açısından önem taşımaktadır.

Anahtar Kelimeler: Alev ağacı, Endofitik Bakteri.

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LIST OF ABBREVIATIONS AND ACRONYMS

<u>Abbreviations</u> <u>and Acronyms</u>	<u>Explanations</u>
%	: Percent
≈	: Approximately
μg	: Microgram
μl	: Microliter
bp	: Base Pairs
Da	: Dalton
g	: Gram
kb	: Kilobase
kDa	: Kilodalton
kg	: Kilogram
L	: Liter
M	: Molar
m	: Meter
mg	: Miligram
mm	: Milimeter
N	: Normal
ng	: Nanogram
nm	: Nanometer
°C	: Centigrade Degree
α	: Alpha
β	: Beta
γ	: Gamma
C	: Carbon
C ₂ H ₄	: Ethylene
Ca(NO ₃)	: Calcium Nitrate
CaCl ₂ .	: Calcium Chloride
CaCO ₃	: Calcium Carbonate

Cd	: Cadmium
CO ₂	: Carbon Dioxide
CoCl ₂	: Cobalt Chloride
CuSO ₄	: Copper Sulphate
FeCl ₃	: Iron Chloride
Fe-EDTA	: Iron EDTA
FeSO ₄	: Iron Sulphate
H	: Hydrogen
H ₂ O ₂	: Hydrogen Peroxide
H ₃ BO ₃	: Boric Acid
HCl	: Hydrochloric Acid
K ₂ HPO ₄	: Dipotassium Hydrogen Phosphate
KAc	: Potassium Acetate
KH ₂ PO ₄	: Potassium Dihydrogen Phosphate
KI	: Potassium Iodide
MgCl ₂	: Magnesium Chloride
MgSO ₄	: Magnesium Sulphate
MnSO ₄	: Manganese Sulphate
N	: Nitrogen
Na ₂ EDTA	: Sodium EDTA
Na ₂ MoO ₄	: Sodium Molybdate
Na ₂ S ₂ O ₃ . 5H ₂ O	: Sodium Thiosulfate
NaCl	: Sodium Chloride
NaOH	: Sodium Hydroxide
NH ₄ NO ₃	: Ammonium Nitrate
NO ₃	: Nitrate
O	: Oxygen
P	: Phosphorus
Pb	: Lead
Zn	: Zinc
ZnSO ₄	: Zinc Sulphate

HDTMA	: Hexadecyltrimethylammonium Bromide
A	: Aerobe
ACC	: 1-aminocyclopropane-1-carboxylate
<i>acdS</i>	: ACC Deaminase Gene
ARA	: Acetylene Reduction Assay
<i>aux</i>	: Auxin Gene
BA	: Benzyladenine
BTB	: Bromothymol Blue
CAS	: Chrome Azurol S
CFU	: Colony Forming Unit
DNA	: Deoxyribonucleic Acid
FA	: Facultative Anaerobe
GA ₃	: Gibberellic Acid
GC	: Gas Chromatography
Gr (-)	: Gram Negative
Gr (+)	: Gram Positive
GTU	: Gebze Technical University
GY	: Glucose-Yeast
IAA	: Indole -3- Acetic Acid
IBA	: Indole Butyric Acid
ISR	: Induced Systemic Resistance
LB	: Luria Bertani
min	: Minute
MPYE	: Mineral Pepton Yeast Extract
NA	: Nutrient Agar
NB	: Nutrient Broth
NFB	: Nitrogen Free Broth
NG	: Nutrient Gelatin
<i>nif</i>	: Nitrogenase Gene
O/F	: Oxidative/Fermentative
o/n	: Overnight

PCR	: Polymerase Chain Reaction
PGPB	: Plant Growth Promoting Bacteria
PGPC	: Plant Growth-Promoting Characteristic
PGRs	: Plant Growth Regulators
ppm	: Parts Per Million
rpm	: Revolutions Per Minute
RT	: Room Temperature
SAM	: S-Adenosylmethionine
SAR	: Systemic Acquired Resistance
SCA	: Simmons' Citrate Agar
SDS	: Sodium Dodecyl Sulfate
sec	: Second
SFC	: Shoot Forming Capacity
SIM	: Sulphite Indole Motile
TAB	: Tryptic Soy Broth
TEM	: Transmission Electron Microscopy
TSA	: Tryptic Soy Agar
U	: Unit
UV	: Ultra Violet
v/v	: Volume/Volume
VBNC	: Viable But Non-Culturable
<i>virD1</i>	: Virulence Gene
w/v	: Weight/Volume

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1. INTRODUCTION

A microorganism is supposed as endophytic when obtained by isolation from plant materials or plants, which are surface sterilized. Having no harmful effect on the plant is one of the main characteristics of endophytic organisms that differs them from pathogens [Hallmann, 2001; Bacon and Hinton, 2002]. The association of endophytic bacteria with plants may be compulsory or facultative. Endophytic bacteria exhibit complex interactions involving mutualism and antagonism with hosts [Parker, 1995]. While the growth of endophytes limited strictly by the plants, endophytes use several mechanisms that enables them to gradually adapt to their hosts' conditions [Dudeja, 2011]. These organisms produce a wide range of compounds, which promote plant growth to keep up stable symbiosis and help plants to better adapt to environmental conditions [Das and Varma, 2009]. Figure 1.1 shows medical, agricultural and environmental applications of endophytic bacteria.

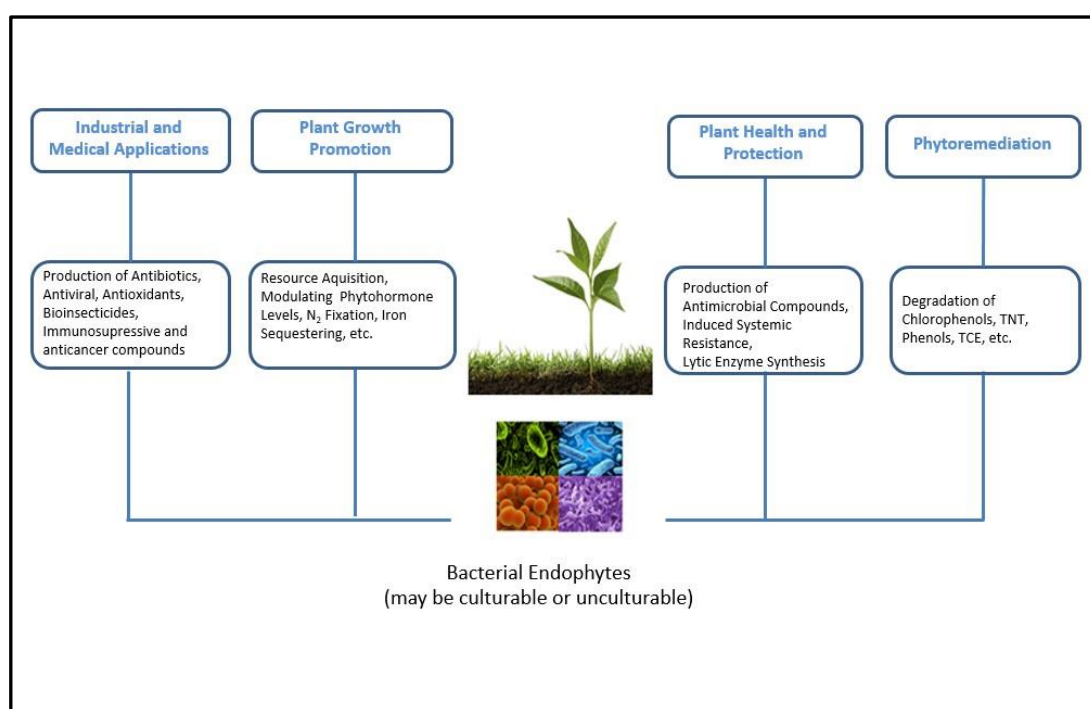


Figure 1.1: Schematic presentation of endophytic bacterial applications.

Endophytic bacteria can promote plant growth through several mechanisms. One of these mechanisms is phytostimulation. Plants need 16 basic elements, mainly

Carbon (C), Hydrogen (H), Nitrogen (N), Oxygen (O), and Phosphorus (P). The plants procure these basic elements for growth and development from the atmosphere, soil, water, and plant growth regulators (PGRs). Endophytes may play a significant role in the transport of these nutrients. Another mechanism is pigment production. Different soil microorganisms produce many commercially important enzymes. Investigations to find different potential sources have resulted in the discovery of several vital enzymes produced by endophytes [Bezerra et al., 2012]. It is also known that the vast majority of endophytes isolated from plants have antimicrobial activity by which they help control microbial pathogens in plants and/or animals. Besides, endophytes are the source of biodegradable materials and new compounds. They can synthesize bioactive compounds used by plants in defense against pathogens. It has also been proven that some of these compounds are useful for the discovery of new drugs. Recent studies have reported hundreds of natural products containing endobacteria-derived alkaloids, terpenoids, and steroids. Among the many biologically active compounds isolated from endophytes are antibiotics, immunosuppressants, anti-cancer agents and biological control agents [Strobel and Daisy, 2003; Joseph and Priya, 2011].

Improvement of endophytic resources can lead to the discovery of new and effective bioactive compounds that cannot be synthesized by chemical reactions. For example, some endophytes can produce siderophores. Siderophores are biologically active compounds with the function of chelating iron ions in organisms. They are used in comprehensive applications in the pharmaceutical and agriculture sectors. It was found that *Metarhizium anisopliae*, an endophytic microorganism isolated from *Taxus chinensis in vitro*, produced a drug called taxol in abundant quantities. Taxol used to treat breast cancer, ovarian cancer, and lung cancer [Liu et al., 2009]. *Colletotrichum gloeosporioides* isolated from the leaves of *Justicia gendarussa* also produce taxol [Gangadevi and Muthumary, 2008]. In addition, endophytes have the ability to break down complex compounds powerfully. For this reason, they are also important in terms of biodegradation and bioremediation. Recently, there has been an increased interest in endophytic bacteria that promote plant growth without damaging the plant, due to the importance of developing "environmentally friendly" technologies and new research areas based on microorganisms.

The presence of an endophytic bacterium has been observed in the Gebze Technical University (GTU), Plant Biotechnology Laboratory after about the fourth year of *in vitro* subculture of fraser photinia (*Photinia X fraseri* Dress.) “Red Robin” plant without any adverse effects on plant health and viability. As “Red Robin” (*Photinia X fraseri* Dress.) is an ornamental, evergreen shrub and has commercial importance with attractive reddish leaves, it is important to characterize this bacterium to reveal out its potential as biofertilizer for not only fraser photinia but also other ornamental plants.

1.1. Aim, Contribution and Scope of the Thesis

The aims of the thesis are:

- Establishment of medium type and culture conditions to allow better growth of endophytic bacterium cultures isolated from *Photinia X fraseri* plants *in vitro* (optimization of medium, growth temperature and incubation period),
- Characterization of the bacterium by standard biochemical tests,
- Biochemical investigation of auxin and cytokinin production in the endophytic strain,
- Molecular analysis of the presence of auxin, cytokinin, nitrogen fixing, and virulence genes in the endophytic bacterium,
- Identification of the effects of endophytic bacterium on *in vitro* proliferation, and rooting of microshoots together with acclimatization of rooted microshoots to *in vivo* conditions.

It is observed previously that the presence of beneficial bacterium fraser photinia “Red Robin” plant *in vitro* microshoots in the GTU Plant Biotechnology Laboratory; extend the subculture period and by this way the plants can protect their vitality for a long time without adding any external additives to the medium. Thus, the presence of beneficial bacterium in the tissue culture medium reduces the frequency of subculture, and prevents labor, material and energy loss. In the scope of the thesis, the microbiological, biochemical and molecular analyzes have been

carried out for not only identification of endophytic bacterium isolated from fraser photinia “Red Robin” *in vitro* cultures but also determination of its positive influences of this bacterium to host plant which could be important for utilization of endophytic bacteria-plant collaboration.

2. LITERATURE REVIEW

2.1. Fraser Photinia “Red Robin” (*Photinia X fraseri* Dress.)

Fraser photinia “Red Robin” (*Photinia X fraseri* Dress.) is an evergreen, hybrid shrub between Japanese *Photinia glabra* and Chinese *Photinia serrulata*. It belongs to Rosaceae family and is a woody perennial plant that can reach 3-5 meters in height and 2-4 meters in width [Larraburu et al., 2007; Lambardi et al., 2013; Llorente and Larraburu, 2013]. Its reddish, impressive leaves that contrast nicely against the dark green mature foliage are resistant to cold. The young red leaves may be seen during spring and autumn seasons [Llorente and Larraburu, 2013]. *Photinia X fraseri* is widely used in many countries for ornamental purposes on the edges of the roads, parks and gardens with these properties and has a commercial significance (Figure 2.1) [Web1, 2015].



Figure 2.1: Fraser photinia “Red Robin” (*Photinia X fraseri* Dress.).

The traditional propagation method of fraser photinia “Red Robin” is rooting the apical cuttings with pulses of phytohormones [Beeson, 2000]. It takes a long time to obtain new plants and the rooting difficulties of cuttings are some limiting factors

for large-scale commercial production of this species [Larraburu et al., 2007]. The large-scale production of ornamental plants such as fraser photinia “Red Robin”, which is traditionally multiplied by grafting, is now achieved through *in vitro* tissue culture methods to clone disease resistant species and to supply target material for gene transfer etc. [Merkle and Dean, 2000; von Aderkas and Bonga, 2000; Ekinici, 2013].

However, frequent resistant bacterial and fungal contaminations have been reported [Boxus and Terzi, 1988; Cassells, 1990]. These contaminants can be epiphytic, endophytic or by accidental contamination [Stead et al., 1998]. Among these, endophytic infections may not be seen, especially in the initial stage of the cultures, but they become visible during subsequent subcultures and cause problems [Thorpe and Harry, 1990].

2.2. Endophytic Microorganisms

It is still not certain how many different species of plants are in the world today. Besides new species are still being identified. However, it is estimated that the total number of plants is about 400,000 species over the world. It is accepted that the vast majority of these species have endophytic organisms.

Endophytes are ubiquitous microorganisms such as bacteria, actinomycetes and fungi that maintain a symbiotic relationship in healthy plant tissues may colonize in all plants and have been isolated from almost all plants examined till date [Wilson, 1995; Nair and Padmavathy, 2014]. There are a few bacterial and fungal endophytic organism examples in Table 2.1. A microorganism is supposed as endophytic when obtained by isolation from plant materials or plants which are surface sterilized. Having no harmful effect on the plant is one of the main characteristics of endophytic organisms that differs them from pathogens [Hallmann et al., 1997; Hallmann, 2001; Bacon and Hinton, 2002; Santoyo et al., 2016].

Table 2.1: Examples of bacterial and fungal endophytic organisms.

Bacteria	Fungi
<i>Pseudomyces viridiflava</i>	<i>Fusarium</i> sp.
<i>Streptomyces griseus</i>	<i>Festuca arundinacea</i>
<i>Serratia marcescens</i>	<i>Rhizoctonia parkeri</i>
<i>Paenibacillus polymyxa</i>	<i>Taxus</i> spp.
<i>Acidovorax facilis</i>	<i>Picea</i> spp.
<i>Rhodococcus rhodochrous</i>	<i>Juniperus</i> spp.

Microorganisms can be firmly attached to the plants, and they can complete a considerable part of their entire life cycle inside plants. Microorganisms that need plant tissues to complete the life cycle are called “obligates” [Hardoim et al., 2015]. On the other hand, some endophytes primarily grow outside of the plant tissues (epiphytes) and occasionally enter the plant endosphere. This group is called “opportunistic endophytes” [Hardoim et al., 2008]. Apart from these two extremes, there is an intermediate group called “facultative endophytes”.

The most common endophytes are commensals whose functions are not yet known in plants. However, these microorganisms, for example, can benefit from the metabolites produced by plants. On the other side, there are also endophytes that benefit the plants. They may be beneficial by promoting plant growth, as well as by providing protection against invading pathogens, via antibiosis or reducing resistance [Hardoim et al., 2015]. Latent pathogens constitute a third group. They have either neutral or harmful effect on the host plant under normal growth conditions. However, these endophytes may be beneficial to plants at different stages of the plant life cycle or under more extreme conditions [Hardoim et al., 2015]. The rarest endophytes are those with positive (mutualistic) or negative (antagonistic) effects on plants [Hallmann et al., 1997].

Because of all these features, more information is needed about the role and importance of endophytes. Many studies have been reported to evaluate endophytes and their effects on plant growth [Salt et al., 1995; Lucy et al., 2004; Badri et al., 2009]. These publications indirectly emphasize the importance of hosts and environment

for endophytes [Thomas et al., 2006]. In addition, there is a growing interest in recent years, as endophytic bacteria, which promote plant growth or have potential applications for plant protection in the direction of the development of "environmentally-friendly" technologies, open new areas based on the use of microorganisms [Compant et al., 2005; Rosenblueth and Martinez-Romero, 2006]. In this context, the isolation and identification of the different endophytes associated with different plants is extremely important.

2.3. Bacterial Endophytes

Bacteria can interact with plants in four ways: as pathogen, symbionts, epiphytes, and endophytes. Among these four types of plant-bacteria associations, endophytic relations are the least studied and less understood associations [Lacava and Azevedo, 2013]. Bacteria, called PGPB (Plant Growth Promoting Bacteria) and promoting plant growth, include free-living bacteria, bacteria that have a symbiotic relationship with plants, cyanobacteria, and bacteria that can colonize in plant tissues (Figure 2.2). Although there are differences between these bacteria, they all use the similar mechanisms to promote plant growth [Glick, 2012; Chaturvedi et al., 2016].

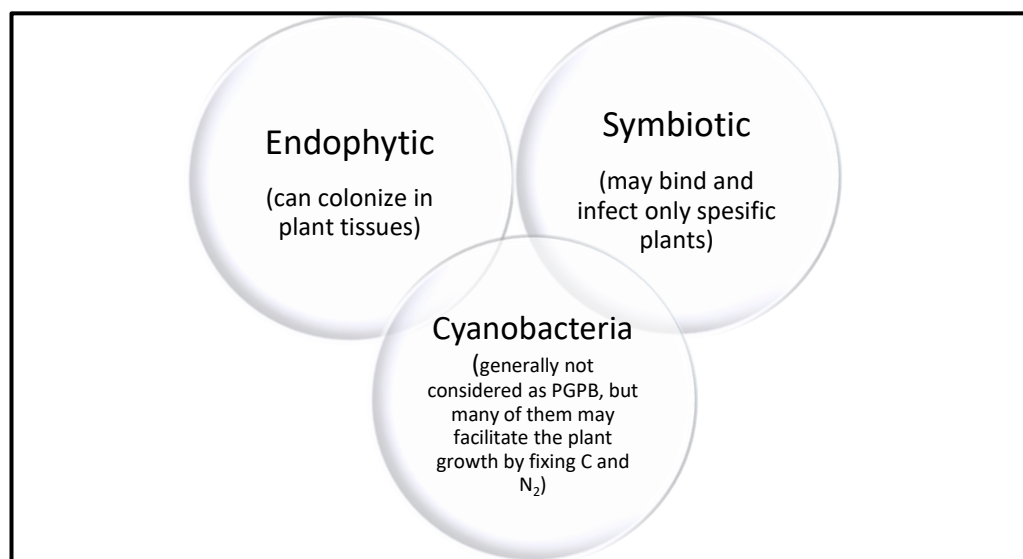


Figure 2.2: Overview of different types of PGPB.

As it is known, plants synthesize various carbonaceous components by reducing the carbon contained in the atmospheric carbon dioxide (CO₂) with the energy they receive from the sunlight in a process called photosynthesis. Through photosynthesis, plants provide nitrogen, carbon etc. to the heterotrophic microorganisms and particularly to the bacteria, which associated to plants. This makes the plants “nutrient reservoirs” for some bacteria. On the other hand, plants also need such bacteria in order to grow and exist in different ecosystems. Briefly, while bacteria benefit from plants for increased nutrient availability, plants also benefit from associated bacteria to reduce stress and increase growth.

Bacterial endophytes are known to last for more than 120 years. According to their life strategies, bacterial endophytes can be classified as “obligate” or “facultative” [Nair and Padmavathy, 2014]. Obligate endophytes are strictly dependent on host plants for their growth and survival. Facultative endophytes thrive outside the host plants at some stage of their life cycle [Hardoim et al., 2008].

Endophytic bacteria exhibit complex interactions involving mutualism and antagonism with hosts [Parker, 1995]. While the growth of endophytes limited strictly by the plants, endophytes use several mechanisms that enables them to gradually adapt to their hosts’ conditions [Dudeja, 2011; Nair and Padmavathy, 2014]. These organisms produce a wide range of compounds, which promote plant growth to keep up stable symbiosis and help plants to better adapt to environmental conditions [Das and Varma, 2009].

The rhizosphere is an actual competitive environment for microorganisms, which are fighting for nutrients and space. Therefore, among the potentially beneficial or pathogenic microorganisms, endophytes, which are the most competitive ones in colonizing plant tissues and in obtaining nutrients, will proliferate in this environment, and will probably have an impact on plant growth and development. Endophytes use different mechanisms to enter plant tissues, especially in plant roots. Any organ of the host plant can be colonized [Schulz and Boyle, 2006]. Bacteria can colonize intracellularly, although they mainly colonize intercellularly. Figure 2.3 is an overview of endophytic bacterial mode of entry into plants [Santoya et al., 2016]. Bacteria are often found in the vascular tissues of the host plant, which is advantageous for distribution [Schulz and Boyle, 2006]. Various endophytic

bacteria colonize different plant tissues. Thereby, some strains colonize only plant roots, while others colonize both roots and stems and others may colonize many parts of the plant such as roots, stems, leaves and even seeds. The most common way that endophytic bacteria use to enter plant tissues is through primary and lateral root cracks and various tissue wounds resulting from plant growth [Santoyo et al., 2016]. Root wounds are attractive entrance sites for bacterial endophytes due to the leakage of plant metabolites, which serve as a nutrient source for bacteria [Hallmann et al., 1997; Lodewyckx et al., 2002]. Other areas that endophytes use to enter the plant include stomata, mainly on young leaves and on young stems; lenticels and germinating radicles. Bacteria can also enter via emergence of lateral roots or root hair cells [Huang, 1986].

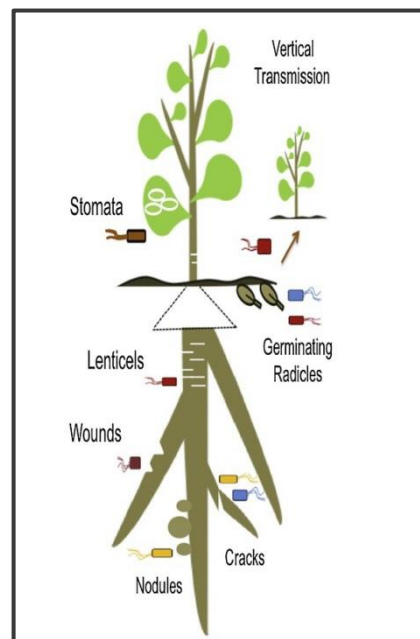


Figure 2.3: An overview of endophytic bacterial mode of entry into plants.

After entering the plant tissue, the endophytic bacteria can localize in the plant tissue it enters, or systematically colonize the plant with active migration or transport through conductive elements or apoplast [Lodewyckx et al., 2002]. Once the endophytes have entered the plant tissue, they can rapidly multiply and generally reach high cell numbers [Hardoim et al., 2008]. In a host plant, endophytic bacteria can be more than one genera and species. However, it is not certain whether the

beneficial effects on the plant are the result of the common activities of these bacterial species [Rosenblueth and Martinez-Romero, 2006].

Although endophytic populations differ in different plants according to many factors, bacterial populations are usually larger in the roots and smaller in the stems and leaves. In addition, the population density of endophytic bacteria found in plants depends on plant species, genotypes and tissues, bacterial growth stage and specialization, differences in the colonization pathway, mutual exclusion of different bacterial populations. Many factors, including season, host plant age, environment and location, change the endophytic biology [Strobel and Daisy, 2003; Lacava and Azevedo, 2013].

Endophytic bacteria have some advantages over rhizospheric colonies; once endophytic bacteria are located in the host tissue, they are not affected by changing soil conditions. These changing conditions, which may inhibit the function and proliferation of rhizospheric bacteria, are temperature changes, soil pH and water contents, and the presence of competing soil bacteria for binding sites on the root surface of the host plant [Glick, 2012; Chaturvedi et al., 2016].

Bacterial endophytes have so far been isolated from monocotyledonous and dicotyledonous plants ranging from woody tree species to herbaceous plants [Lodewyckx et al., 2002]. Microbial contamination, either implicitly or explicitly, can be a source of endophytes. A number of culture-dependent and culture-independent methods are used to isolate endophytic bacteria [Nair and Padmavathy, 2014; Chaturvedi et al., 2016]. In culture-dependent methods, plant parts are cultured in appropriate medium for bacteria following surface sterilization. Bacterial contamination is expected to occur in the first few days after *in vitro* tissue culture methods. Bacterial contamination following long-term subculturing is predicted as endophytic. Culture-independent molecular approaches based on 16S rRNA gene analysis. Culture-independent analysis of bacterial populations in plants suggests that bacterial endophytic populations are much more diverse than formerly realized [Lacava and Azevedo, 2013]. Various studies on endophytic bacteria in plants have shown that the use of fingerprinting techniques and clone analysis may provide additional information for analyzing the community composition of endophytic bacteria. The presence of a large proportion of Gram-positive and Gram-negative

bacteria, including *Enterobacter*, *Pseudomonas*, *Staphylococcus*, *Xanthomonas*, *Agrobacterium* and *Methylobacterium* spp., in tissue culture of different plant species have been reported [Leifert and Cassells, 2001; Herman, 2004; Kulkarni et al., 2004; , Thomas et al., 2006; Thomas, 2007]. Many endophytes, such as *Pseudomonas*, *Burkholderia* and *Bacillus*, are general soil bacteria [Lodewyckx et al., 2002]. The endophytic bacteria isolated to date mainly belong to Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes [Rosenblueth and Martinez-Romero, 2006; Chaturvedi et al., 2016]. In general, Phylum Proteobacteria, including α , β and γ -Proteobacteria classes, have been reported to dominate in the diversity analysis of endophytes. However, Firmicutes and Actinobacteria members are among the most consistent endophytes classes found in plants. Other classes, such as Bacteroidetes, Planctomycetes, Verrucomicrobia and Acidobacteria are less common in plants as endophytes [Santoyo et al., 2016]. Other common bacterial endophytic genera are *Pseudomonas*, *Bacillus*, *Microbacterium*, *Burkholderia*, *Stenotrophomonas*, *Micrococcus* and *Pantoea* [Hallmann et al., 1997; Chaturvedi et al., 2016; Santoyo et al., 2016]. Bacterial genera reported to be endophytic are listed in Table 2.2 according to their gram staining, shape and oxygen requirements. All these bacterial endophytic genera are also commonly found in the rhizosphere. For this reason, it is suggested that the endophytic microbiome may be a subpopulation of the rhizosphere inhabitants [Santoyo et al., 2016]. Although the presence of bacteria in microbial plants is often considered as microbial contamination to be avoided and eliminated [George et al., 2008]; the relationship between beneficial endophytic bacteria and microbial plants may have positive effects on microbial growth [Dias et al., 2009].

Table 2.2: Some of endophytic bacterial genera according to their gram staining, oxygen requirements, shape and class.

Genera	Gram Staining	Oxygen Requirements	Shape	Class
<i>Acidovorax</i>	Gr (-)	Aerobic	Slightly curved rods	Proteobacteria
<i>Acinetobacter</i>	Gr (-)	Obligate Aerobic	Rod-shaped	Gammaproteobacteria
<i>Actinomyces</i>	Gr (+)	Facultative Anaerobic	Rod-shaped	Actinobacteria
<i>Aeromonas</i>	Gr [-]	Facultative Anaerobic	Rod-shaped	Gammaproteobacteria
<i>Agrobacterium</i>	Gr (-)	Variable	Rod-shaped	Alphaproteobacteria
<i>Alcaligenes</i>	Gr (-)	Aerobic	Rod-shaped	Betaproteobacteria
<i>Alcanivorax</i>	Gr (-)	Aerobic	Rod-shaped	Gammaproteobacteria
<i>Alteromonas</i>	Gr (-)	Aerobic	Curved rods	Gammaproteobacteria
<i>Aminobacter</i>	Gr (-)	Aerobic	Rod-shaped	Alphaproteobacteria
<i>Aquaspirillum</i>	Gr (-)	Aerobic	Helical	Betaproteobacteria
<i>Arthrobacter</i>	Gr (+)	Aerobic	Rod, Cocci	Actinobacteria
<i>Azomonas</i>	Gr (-)	Aerobic	Oval or Spherical	Gammaproteobacteria
<i>Azotobacter</i>	Gr (-)	Aerobic	Oval or Spherical	Gammaproteobacteria
<i>Azospirillum</i>	Gr (-)	Microaerophilic	Rod-shaped	Alphaproteobacteria
<i>Bacillus</i>	Gr (+)	Obligate Aerobic /Facultative Anaerobic	Rod-shaped	Bacilli
<i>Beijerinckia</i>	Gr (-)	Aerobic	Slightly curved rods	Alphaproteobacteria
<i>Blastobacter</i>	Gr (-)	Aerobic	Pleomorphic	Alphaproteobacteria
<i>Blastomonas</i>	Gr (-)	Obligate Aerobic	Rod-shaped / Ovoid	Alphaproteobacteria
<i>Brachymonas</i>	Gr (-)	Aerobic	Rod-shaped	Betaproteobacteria
<i>Brenneria</i>	Gr (-)	Facultative Anaerobic	Rod-shaped	Gammaproteobacteria
<i>Burkholderia</i>	Gr (-)	Obligate Aerobic	Rod-shaped	Betaproteobacteria
<i>Chromobacterium</i>	Gr (-)	Aerobic	Rod-shaped	Betaproteobacteria

Table 2.2: Continued.

<i>Comamonas</i>	Gr (-)	Aerobic	Rod-shaped	Betaproteobacteria
<i>Corynebacterium</i>	Gr (+)	Aerobic	Rod-shaped	Eubacteria
<i>Derxia</i>	Gr (-)	Obligate Aerobic	Rod-shaped	Betaproteobacteria
<i>Devosia</i>	Gr (-)	Aerobic	Rod-shaped	Alphaproteobacteria
<i>Enterobacter</i>	Gr (-)	Facultative Anaerobic	Rod-shaped	Gammaproteobacteria
<i>Flavobacterium</i>	Gr (-)	Aerobic	Rod-shaped	Flavobacteria
<i>Flexibacter</i>	Gr (-)	Strictly Aerobic / Facultative Anaerobic	Rod-shaped	Sphingobacteria
<i>Frankia</i>	Gr (+)	Aerobic	Rod-shaped	Actinobacteria
<i>Halomonas</i>	Gr (-)	Anaerobic	Rod-shaped	Gammaproteobacteria
<i>Mesorhizobium</i>	Gr (-)	Aerobic	Pleomorphic	Alphaproteobacteria
<i>Ochrobactrum</i>	Gr (-)	Strictly Aerobic	Rod-shaped	Alphaproteobacteria
<i>Pantoea</i>	Gr (-)	Aerobic	Rod-shaped	Gammaproteobacteria
<i>Phyllobacterium</i>	Gr (-)	Aerobic	Rod-shaped / Ovoid	Alphaproteobacteria
<i>Photobacterium</i>	Gr (-)	Anaerobic	Rod-shaped	Gammaproteobacteria
<i>Pseudoalteromonas</i>	Gr (-)	Aerobic	Curved rods	Gammaproteobacteria
<i>Pseudomonas</i>	Gr (-)	Aerobic	Rod-shaped	Gammaproteobacteria
<i>Psychrobacter</i>	Gr (-)	Aerobic	Cocci	Gammaproteobacteria
<i>Rhizobacter</i>	Gr (-)	Aerobic	Rod-shaped	Rhizobacteria
<i>Rhizobium</i>	Gr (-)	Aerobic / Microaerophilic	Rod-shaped	Alphaproteobacteria
<i>Rhodococcus</i>	Gr (+)	Aerobic	Cocci	Actinobacteria
<i>Sphingomonas</i>	Gr (-)	Strictly Aerobic	Rod-shaped	Alphaproteobacteria
<i>Spirillum</i>	Gr (-)	Microaerophilic	Spiral-shaped	Betaproteobacteria
<i>Streptomyces</i>	Gr (+)	Aerobic	Filamentous	Actinobacteria
<i>Vibrio</i>	Gr (-)	Facultative Anaerobic	Curved rod- shaped	Gammaproteobacteria
<i>Xanthomonas</i>	Gr (-)	Strictly Aerobic	Rod-shaped	Gammaproteobacteria
<i>Zoogloea</i>	Gr (-)	Aerobic	Rod-shaped	Betaproteobacteria
<i>Methylobacterium</i>	Gr variable	Obligate aerobic	Rod-shaped	Alphaproteobacteria

In nature, some endophytic bacteria useful for the host plant may naturally be present in the soil, may pass to the plant, are transported to organs growing on the soil, and form colonies on them. Bacterial endophytes may increase the plant's ability to find nutrients and gain more resistance to plant abiotic and/or biotic stresses through regulation of the hormones [Kim et al., 2012]. Endophytic microorganisms may promote plant growth by using a number of different mechanisms such as phosphorus solubilization [Wakelin et al., 2004], indole acetic acid production [Lee et al., 2004] and production of siderophores [Costa and Loper, 1994]. In addition, endophytic organisms can also provide essential vitamins to plants [Pirttila et al., 2004]. A number of beneficial effects on plant growth, such as osmotic regulation, regulation of stomata, modification of root morphology, increased transport of minerals, and alteration of nitrogen accumulation and metabolism, are also associated with endophytes [Compant et al., 2005]. Endophytic bacteria can protect plants from fungal, microbial and insect infections by producing chitinases [Krechel et al., 2002], mannitol dehydrogenase [Jennings et al., 1998], volatile organic compounds and other molecules with antimicrobial activity [Mendes et al., 2007], and stimulating ultra-structural modifications that inhibit the passage of plant pathogens [Benhamou et al., 1996].

2.4. Plant Growth Promoting Mechanisms

Despite the debate as to whether or not living in plant tissues is an advantage for bacterial endophytes, there are numerous benefits of bacterial endophytes; primarily plant growth promotion and protection against pathogens. In addition, bacterial endophytes correlate more effectively with plants than with rhizospheric bacteria under different environmental conditions [Rosenblueth and Martinez-Romero, 2006; Santoyo et al., 2016]. Thus, bacterial endophytes have a better chance of showing direct beneficial effect to the plant.

The promotion of plant growth by microorganisms can result in bacterial nitrogen fixation, phytohormone production, increased availability of minerals, and bacterial control of phytopathogens in the root zone of the plant (antifungal or

antibacterial agent production, siderophore production, competition for food and host resistance or immunity).

The ability of various bacterial endophytes to promote plant growth occurs because of different mechanisms as other bacteria that promote plant growth. Plant growth occurs either through the supply of the necessary nutrients mainly nitrogen, phosphorus and iron or modulating the hormone levels of the plant by the endophytes. Modulation of hormone levels is mediated by endophytes synthesizing phytohormones such as auxin, cytokinin and gibberellin. In addition, some endophytes may decrease the level of ethylene, a phytohormone, by synthesizing 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an enzyme that cleaves ACC, the precursor of ethylene in all higher plants. When an endophyte reduces the plant damage after infection caused by some soil fungi and bacteria, this situation may be called biocontrol activity of bacteria [Glick, 1995; Glick, 2012; Chaturvedi et al., 2016; Santoyo et al., 2016]. It is known that endophytes produce a variety of secondary metabolites including antibiotics, anticancer compounds, volatile organic compounds, antifungal, antiviral, insecticidal and immunological agents. Although many biologically active compounds are currently isolated from endophytic organisms, many of the products that have not yet been discovered are of importance as a source of natural products [Ryan et al., 2008].

Briefly, plant growth promotion occurs by three ways (Figure 2.4); (i) endophytes may facilitate the acquisition of resources including nitrogen, phosphorus and iron from environment; (ii) endophytes may modulate plant growth by providing or regulating various plant hormones including auxin, cytokinin, or ethylene; or (iii) endophytes may protect plants from pathogens by producing various compounds.

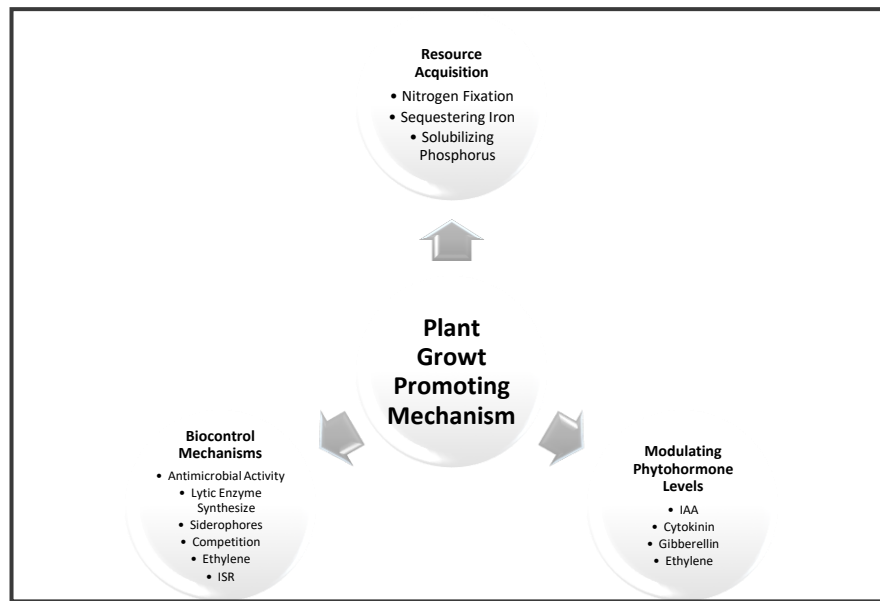


Figure 2.4: Plant growth mechanisms of bacterial endophytes.

2.4.1. Resource Acquisition

Both plants and soil bacteria use the nutrients they receive from the soil to grow. Plants need essential elements such as C, H, N, O, and P, as well as a number of other elements [Nair and Padmavathy, 2014]. In addition to different metals in small quantities, plants and bacteria also require fixed nitrogen, iron, and phosphorus. One of the most important benefits of bacterial endophytes is that they facilitate the plant's acquisition of resources that they lack from the soil. It is the best-studied mechanisms of bacterial plant growth promotion.

2.4.1.1. Nitrogen Fixation

One of the main nutrients that is necessary for all living organisms to grow is nitrogen. Although nitrogen is abundant in the atmosphere (about 80%), it must be reduced to ammonia by plants before it can be metabolized and participated in the structure of proteins, nucleic acids and other biological molecules. A high amount of energy is required for this conversion because the triple bond of the nitrogen is highly stable [Glick, 2015].

Nitrogen-fixing bacteria provide nitrogen to the plant to prevent problems caused by nitrogen deficiency. In a study, wheat (*Triticum aestivum*) is inoculated with nitrogen fixing bacterium, *Klebsiella pneumoniae* 342 [Iniguez et al., 2004]. It is observed that strain relieved nitrogen deficiency symptoms and increased total nitrogen concentration in plant. This characteristic of nitrogen fixing bacteria, promises to be a viable and cheap alternative to chemical fertilizer, which is hazardous to environment and human health, used in agriculture or forestry.

Nitrogenases consist of two proteins that can be purified separately: dinitrogenase and dinitrogenase reductase. The dinitrogenase, also known as MoFe protein or component 1, is a tetramer of 220 to 240 kDa of the *nifD* and *nifK* genes products. The dinitrogenase reductase, also called Fe protein or component 2, is a 60 kDa dimer of the *nifH* gene product [Rajeswari and Mangai-Kasthuri, 2009].

Initial studies for *nif/fix* genes in endophytes have been focused on encoding structural genes for nitrogenase subunits, nitrogenase enzyme maturation, cofactor biosynthesis, *nif*-gene regulation, and electron transfer functions. These studies and the sequencing of adjacent regions revealed other genes [Reis et al., 2007]. Nitrogenase (*nif*) genes required for nitrogen fixation include structural genes, genes that play a role in the activation of the Fe protein, iron molybdenum cofactor biosynthesis, electron donation, and regulatory genes necessary for enzyme synthesis and function. In diazotrophic bacteria, the *nif* genes typically reside in a cluster of about 20-24 kb, and seven operons encode 20 different proteins. Due to the complexity of this system, it is difficult to develop genetic strategies to improve nitrogen fixation [Glick, 2012].

2.4.1.2. Sequestering Iron

Iron is the fourth most abundant element on earth and all living organisms require iron as a component of proteins involved in important processes such as respiration, photosynthesis, and nitrogen fixation [Glick, 2012; Ahmed and Holmström, 2014; Glick, 2015]. However, either bacteria or plants in aerobic soils cannot assimilate it. Because the most common form of iron in aerobic soils, ferric ion or Fe^{+3} , is only slightly soluble and can't be readily transport into cells [Neilands,

1995]. For this reason, the amount of iron available for assimilation by living organisms is extremely low. Both microorganisms and plants require iron at high levels. However, getting enough iron is even more problematic in the rhizosphere where plants, bacteria and fungi compete for iron. In order to survive with such a limited iron source, some soil bacteria may synthesize low molecular weight siderophores ($\approx 400\text{-}1500$ Da), which have extraordinarily high affinity for Fe^{+3} and facilitate iron uptake [Glick, 1995; Glick, 2012; Lacava and Azevedo, 2013; Ahmed and Holmström, 2014]. Siderophore synthesizing bacteria takes up iron by using an outer cell membrane receptor that is specific for this complex. Some bacteria produce hydroxamate-type siderophores, while others produce catecholate-types [Neilsen, 1995]. Many plants have mechanisms to bind the bacterial iron-siderophore complex, transport it through the plant, and then release the iron from the siderophore, so that it can be utilized by the plants [Glick, 1995; Lodewyckx et al., 2002; Glick, 2012].

2.4.1.3. Solubilizing Phosphorus

Phosphorus is an essential macro-element, required for all living organisms including plants. Phosphorus is one of the crucial growth-limiting elements in plants. Plants can take up phosphorus only in monobasic or dibasic soluble forms. It is present in the soil as organic compounds and as minerals (inorganic form). In general, the amount of phosphorus in the soil is high (between 400 and 1200 mg/kg of soil). It participates in metabolic processes in plants such as photosynthesis, energy transfer and synthesis and breakdown of carbohydrates. It also promotes root development, seed formation, early flowering, and tillering [Rodriguez and Fraga, 1999; Yadav and Verma, 2012; Lacava and Azevedo, 2013]. Nevertheless, the amount of readily available phosphorus for plants is very low compared with the total amount of phosphorus in the soil (normally at levels of 1 ppm or less). Plants can only take up phosphorus in soluble form, so plant growth will be limited when soil soluble phosphorus is inadequate [Rodriguez and Fraga, 1999]. Among all the microbial populations in the soil, P-solubilizing bacteria generally constitute from 1 to 50% of the total population while P-solubilizing fungi constitute only 0.1-0.5% of the total population [Chen et al., 2006]. Thus, the solubilization and mineralization of

phosphorus in the soil is an important mechanism that bacterial endophytes use to promote plant growth. Generally, the solubilization of inorganic phosphorus occurs by the action of low molecular weight organic acids, such as gluconic and citric acid, which are synthesized by various soil bacteria. On the other hand, the organic phosphorus mineralization occurs by synthesis of various phosphatases, which catalyze the hydrolysis of phosphoric esters. Phosphate solubilization and mineralization can coexist in the same bacterial strain [Glick, 2012; Lacava and Azevedo, 2013]. The ability of endophytes to solubilize phosphorus is an important component of plant growth promoting capability because endophytic bacteria are compatible with host plants and can colonize the tissues of host plants without being identified as pathogens [Rosenblueth and Martinez-Moreno, 2006, Lacava and Azevedo, 2013].

There have been many studies investigating the phosphorus solubilizing ability of soil bacteria and their growth characteristics in various environmental conditions. Among the isolated bacteria, it may be suggested that those capable of solubilizing phosphorus and able to withstand extreme conditions such as wide range of pH, high temperatures and salinity are promising as fertilizers. For example, Malboobi and his colleagues (2009) isolated 22 phosphorus solubilizing bacterial strain from soil samples. They found that three of them, *Pantoea agglomerans* strain P5, *Microbacterium laevaniformans* strain P7 and *Pseudomonas putida* strain P13, possessed this ability.

2.4.2. Modulating Phytohormone Levels

PGRs are naturally occurring organic compounds that affect physiological processes. When they are produced endogenously in plants, they are called "plant hormones" or "phytohormones". Term of PGRs is used by the agricultural chemical industry for synthetic compounds with plant growth regulatory properties [Arshad and Frankenberger, 1997]. Plant hormones play an important role in plant growth and development and in the response of plants to the environment. Moreover, during the lifetime of a plant, a number of nonlethal stress factors can limit its growth. This situation continues until either the plant is able to adjust its metabolism

to overcome the effects of stress or the stress factor is removed. Plant regulates endogenous phytohormone levels to overcome negative effects of plant stress conditions. However, this strategy is not always successful. Bacterial endophytes found in the rhizosphere may also produce or modulate phytohormones. Bacterial endophytes may alter the phytohormone levels to affect the plant's hormone balance and the plant's response to stress [Glick, 2012]. Table 2.3 shows the functions of phytohormones [Glick, 2015], and some PGRs producer bacterial strains are showed in Table 2.4 [Glick 2012; Glick, 2015].

Table 2.3: Functions of plant hormones.

IAA	Cytokinin	Gibberellin	Ethylene
<i>Differentiation of vascular tissue, Root and shoot growth to light and gravity, Apical dominance, Initiation of lateral and adventitious roots, Stimulation of cell division, Elongation of stems and roots</i>	<i>Modulation of cell division, Seed germination, Apical dominance, Root elongation, Differentiation of xylem and chloroplast, Transition to reproductive growth phase, Flower and fruit development, Leaf senescence, Nutritional signaling, Plant-pathogen interactions</i>	<i>Stem growth, Germination dormancy, Flowering, Leaf and fruit senescence</i>	<i>Seed germination, Formation of root and shoot primordia, Root branching and elongation, Tissue differentiation, Lateral bud development, Flowering initiation, Flower opening and senescence, Fruit ripening and degreening, Anthocyanin synthesis, Leaf and fruit abscission, Storage product hydrolysis, Production of volatile organic compounds</i>

Table 2.4: Some soil bacteria that are able to synthesize phytohormones.

IAA	Cytokinin	Gibberellin	Ethylene
<i>Pseudomonas sp.</i> , <i>Bacillus sp.</i> , <i>Aspergillus niger</i> , <i>Rhizopus</i> , <i>Acetobacter diazotrophicus</i> , <i>Herbaspirillum seropedicae</i> , <i>Rhizobium sp.</i> , <i>Azospirillum</i> <i>sp.</i> , and <i>Erwinia sp.</i> , <i>Brevibacterium</i> , <i>Klebsiella</i> , <i>Enterobacter</i> , <i>Alcaligenes</i> <i>faecalis</i> , <i>Paenibacillus</i> , <i>Pseudomonas</i> , <i>Streptomyces</i> , <i>Citrobacter</i> , <i>Pantoea</i> , <i>Methylobacterium</i> , <i>Microbacterium</i> , <i>Micromonospora</i> , <i>Tsukamurella</i> , etc.	<i>Azotobacter spp.</i> , <i>Rhizobium</i> <i>spp.</i> , <i>Bacillus subtilis</i> , <i>Rhodospirillum rubrum</i> , <i>Paenibacillus polymyxa</i> , <i>Pseudomonas fluorescens</i> , <i>Pseudomonas agglomerans</i> , etc.	<i>Acetobacter diazotrophicus</i> , <i>Acinoetobacter calcoaceticus</i> , <i>Agrobacterium tumefaciens</i> , <i>Agrobacterium radiobacter</i> , <i>Arthrobacter atrocyaneus</i> , <i>Arthrobacter aurescens</i> , <i>Bacillus brevis</i> , <i>Bacillus cereus</i> , <i>Bacillus aquimaris</i> , <i>Azotobacter chroococcum</i> , <i>Azospirillum brasilense</i> , <i>Arthrobacter citrens</i> , <i>Arthrobacter globiformis</i> , etc.	<i>Pseudomonas fluorescens</i> , <i>Pseudomonas putida</i> , <i>Azospirillum spp.</i> , <i>Rhizobium</i> <i>spp.</i> , <i>Agrobacterium spp.</i> , <i>Achromobacter spp.</i> , <i>Burkholderia spp.</i> , <i>Ralstonia</i> <i>spp.</i> , <i>Enterobacter spp.</i> ,

2.4.2.1. Indole -3- Acetic Acid (IAA)

Auxin is the first isolated and characterized phytohormone. Although there are many natural auxins described in the literature, Indole-3-acetic acid (IAA) is the most studied and most common auxin among them (Figure 2.5) [Web2, 2013]. IAA is mainly synthesized from tryptophan or indole in leaf primordia and young leaves. Most of the various microorganisms living in the rhizosphere are known to synthesize IAA [Patten and Glick, 1996]. Plant-associated bacteria can synthesize many plant hormones, but IAA can be produced by 80% of plant-associated bacteria [Lacava and Azevedo, 2013]. IAA affects plant cell division, extension, and differentiation; stimulates seed and tuber germination; increases the rate of xylem and root development; controls processes of vegetative growth; initiates lateral and adventitious root formation; mediates responses to light, gravity and florescence; affects photosynthesis, pigment formation, biosynthesis of various metabolites, and resistance to stressful conditions [Zhao, 2010; Glick, 2012].

The effect of IAA on plant growth is obvious, but why does the bacteria produce this growth hormone? By stimulating plant development, these microorganisms can also increase the production of plant metabolites that they can use for their growth. However, there are other benefits of IAA that include detoxification of tryptophan analogs that are detrimental to bacterial cells or inhibition of plant defense enzymes, chitinase and β -1,3-glucanase. Therefore, IAA production will facilitate invasion of plants by bacteria [Patten and Glick, 1996]. IAA-synthesizing bacteria include *Pseudomonas* sp., *Bacillus* sp., *Aspergillus niger*, *Rhizopus*, *Acetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Rhizobium* sp., *Azospirillum* sp., and *Erwinia* sp.

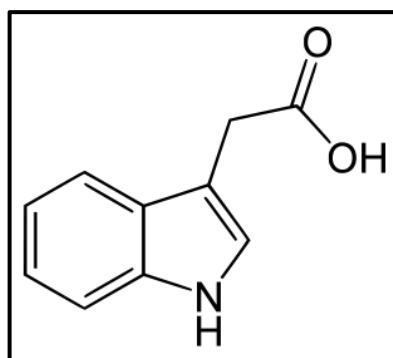


Figure 2.5: Chemical structure of indole -3- acetic acid (IAA).

There are three types of associations between plant and bacteria, based on auxin. (i) Bacterial IAA genes can be directly transferred to the host plant genome, such as in the majority of the *Agrobacterium* species; (ii) the bacterium can live in plant tissues and secrete the IAA into surrounding tissues; (iii) the bacteria can live outside of the host plant, and may provide IAA to plant by excreting [Patten and Glick, 1996].

Different IAA concentrations affect the physiology of plants in dramatically different ways. Some plants may be more or less sensitive to IAA than others. In many cases (e.g., root development) auxin has an inhibitory effect at high concentrations. The plant IAA level can be altered by the acquisition of IAA secreted by bacterial endophytes. In this context, the determination of the level of IAA synthesized by the plant is important in terms of understanding whether the bacterial IAA stimulates or suppresses plant growth [Patten and Glick, 1996; Glick, 2012].

Azospirillum brasilense SP6 mutant, *Azospirillum brasilense* SpM798 is a very low IAA producer compared to wild type strain. Barbieri et al., (1991) inoculated wheat seed with both wild type and mutant strain. Results showed that mutant strain has a reduced ability in promoting root development compared to IAA producing wild type strain. In addition, inoculation with mutant strain causes a decrease in mineral uptake by plants.

Plant hormones such as auxin are in interaction with other phytohormones. For example, IAA regulates the expression of ACC synthase enzyme, which catalyzes the conversion of S-Adenosylmethionine (SAM) to ACC, the precursor of ethylene. Therefore, if the level of IAA in plant tissues is high, the level of ethylene is expected

to increase [Theologies, 1986]. Ethylene, as described in 2.4.2.4, is a plant hormone that prevents prolongation and promotes senescence in growing tissues. Auxin also regulates the biosynthesis of cytokinins [Nordström et al., 2004].

2.4.2.2. Cytokinins

Cytokinin, a phytohormone, controls many physiological events as well as cell division or cytokinesis, in plant roots and shoots. This name is given because of its ability to promote cytokinesis. Cytokinins are mainly related to cell growth and differentiation, but also affect apical dominance, xylem and chloroplast differentiation, nutritional signaling, axillary bud growth and leaf senescence. The presence of cytokinins at high levels in the plant can delay plant senescence that occurs as a direct consequence of stress, such as high salt levels and drought [Glick, 2015].

Natural cytokinins can be classified as adenine-type (kinetin, zeatin, and 6-benzylaminopurine) cytokinins and phenylurea-type (diphenylurea and thidiazuron) cytokinins. Only adenine-type cytokinins are found in plants and most of these types' cytokinins are synthesized in plant roots [Mok and Mok, 2001]. Cytokinins are also synthesized in actively dividing tissues such as cambium [Chen et al., 1985]. Although the first discovered cytokinin is kinetin, the most common cytokinin in plants is zeatin, which isolated from corn (*Zea mays*), (Figure 2.6) [Web2, 2013]. Since kinetin is not actually produced in plants but is derived from yeast extract, it is now thought of as synthetic cytokinin [Davies, 1987].

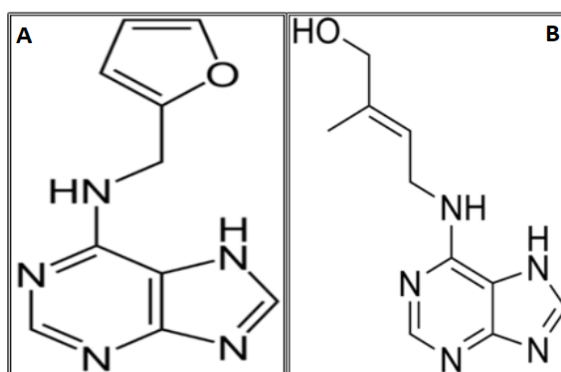


Figure 2.6: Chemical structure of A) kinetin and B) zeatin.

Cytokinins work in concert with auxin, another plant growth hormone, and these two hormones are complementary to each other with opposite effects. In the effect of cytokinin on plant growth, the ratio of auxin to cytokinin plays an important role.

Certain bacteria may also secrete free cytokinins. Naturally occurring cytokinins can be found in some bacteria such as *Azotobacter* spp., *Rhizobium* spp., *Bacillus subtilis*, *Rhodospirillum rubrum*, and *Paenibacillus polymyxa*. Bacterial endophytes secrete and produce cytokinins in significant amounts or cause hormone synthesis of plant cells. It stimulates plant tissues to grow that result in infection with these organisms.

Some phytopathogens have also been found to produce cytokinins. It is thought that beneficial bacteria produce cytokinin lower levels than phytopathogens and thus endophytic bacteria have a stimulatory effect on plant growth whereas phytopathogens are over stimulatory to plant growth [Glick, 2015].

2.4.2.3. Gibberellins

Gibberellins were discovered in Japan during World War II. It was first isolated in 1935 from fungal strain *Gibberella fujikuroi* and named as gibberellin. Over the last 40 years, more than 100 gibberellins have been found naturally synthesized in plants. These are called GA₁, GA₂, and GA₃ etc. in order of discovery. Gibberellic acid (GA₃) is the first characterized and most studied gibberellin (Figure 2.7) [Web2, 2013]. Biologically active GAs are only GA₁, GA₃, GA₄, and GA₇.

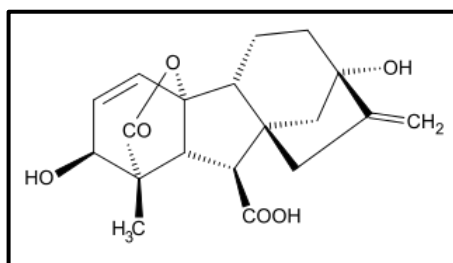


Figure 2.7: Chemical structure of gibberellic acid (GA₃).

Gibberellins are plant hormones that regulate growth and influence various developmental processes such as seed germination, seedling emergence, regulation of vegetative and reproductive bud dormancy, inhibition of floral bud differentiation in woody angiosperms, stem elongation, leaf and root growth, root hair abundance, flowering, sex expression, enzyme induction, and leaf and fruit senescence during almost all stages of plant growth and can be classified as tetracyclic diterpenoid acids with *ent*-gibberallane backbone [Davies, 1987; Bottini et al., 2004; Spaepen, 2015]. In most of these processes, gibberellins act together with other plant hormones and additional regulatory factors.

Gibberellins are not only produced by plants and fungi but also by bacteria [Atzorn et al., 1988; Bottini et al., 1989; Janzen et al., 1992; Bastian et al., 1998; Gutierrez-Manero et al., 2001; Bottini et al., 2004]. Gibberellins do not have a known role in fungi and bacteria, but rather they appear to be secondary metabolites that can act as signaling factors toward the host plant [Bottini et al., 2004]. Gibberellins are the least studied plant hormones due to difficulty of their extraction and analytic detection [Arshad and Frankenberger, 1997].

2.4.2.4. Ethylene

It was discovered in the late nineteenth century by the Russian scientist Dmitri Nelyubov that ethylene was a biologically active compound [Abeles et al., 1992]. It is now known that ethylene is an important phytohormone that plays a role in regulating almost every aspect of plant growth. All organs in high plants can synthesize ethylene, whether the tissues are healthy or diseased, even though the highest ethylene production occurs in senescing tissues or in ripening fruits [Ma et al., 2003]. As a plant hormone, ethylene has a wide range of biological activity and it is active even at low concentrations such as 0.05 $\mu\text{l/L}$ [Abeles et al., 1992]. Ethylene, which is produced in almost all plants, can effect plant growth and development in a number of different ways and has a central role in cellular metabolism [Hardoim et al., 2008]. Ethylene is involved in seed germination, tissue differentiation, formation of root and shoot primordia, root branching and elongation, lateral bud development, flowering initiation, flower opening and senescence, anthocyanin

synthesis, fruit ripening and degreening, storage product hydrolysis, leaf senescence, leaf and fruit abscission, activating the synthesis of other plant hormones, rhizobia nodule formation, mycorrhizae-plant interaction, and the response of plants to both biotic and abiotic stresses [Abeles et al., 1992; Glick, 2012; Glick, 2015].

Stress conditions such as flood, drought, the presence of toxic metals and organic pollutants, radiation, injury, insect predation, high salt, extreme temperatures, and the presence of various pathogens, including viruses, bacteria and fungi, increase the ethylene level that the plant synthesizes [Lodewyckx et al., 2002; Glick et al., 2007; Glick, 2012]. Ethylene can trigger the initiation of senescence, chlorosis and abscission processes and ultimately lead to plant death in high concentrations [Abeles et al., 1992; Nascimento et al., 2012]. Bacteria regulate the level of ethylene in the plant by two different mechanisms: (i) by cleaving ACC to its constituents or (ii) by inhibiting ACC synthase enzyme activity [Hardoim et al., 2008]. Many studies have shown that ACC deaminase enzyme synthesis, which has no known function in bacteria, is a common feature of PGPB [Lodewyckx et al., 2002; Glick, 2012]. IAA, which is synthesized by bacterial endophytes, may stimulate plant growth together with endogenous plant IAA, as well as induce the synthesis of plant enzyme ACC synthase. ACC synthase, in all the higher plants, converts SAM to ACC, the precursor component of ethylene. Some of this newly synthesized ACC is secreted from plant seeds and roots. This ACC is taken by endophytes and converted by the bacterial ACC deaminase enzyme into readily assimilable components such as ammonia and α -ketobutyrate. As a direct consequence of this enzyme activity, there is a decrease in the ethylene amount of produced by the plant. ACC oxidase (Aminocyclopropane Carboxylate Oxidase) is an enzyme that synthesizes ethylene using oxygen and ACC. Some of the ACC is converted to ethylene by ACC oxidase (Figure 2.8; Arshad et al., 2007). Thus, the endophytes that produce ACC deaminase prevents the plant ethylene levels from reaching inhibitory levels of plant growth [Glick et al., 2007; Glick, 2012]. ACC deaminase may increase plant's fitness by reducing the ethylene level in developing or stressed plants. For this reason, ACC deaminase production can be considered as a plant growth-promoting characteristic (PGPC) [Lodewyckx et al., 2002; Ma et al., 2003; Glick et al., 2007].

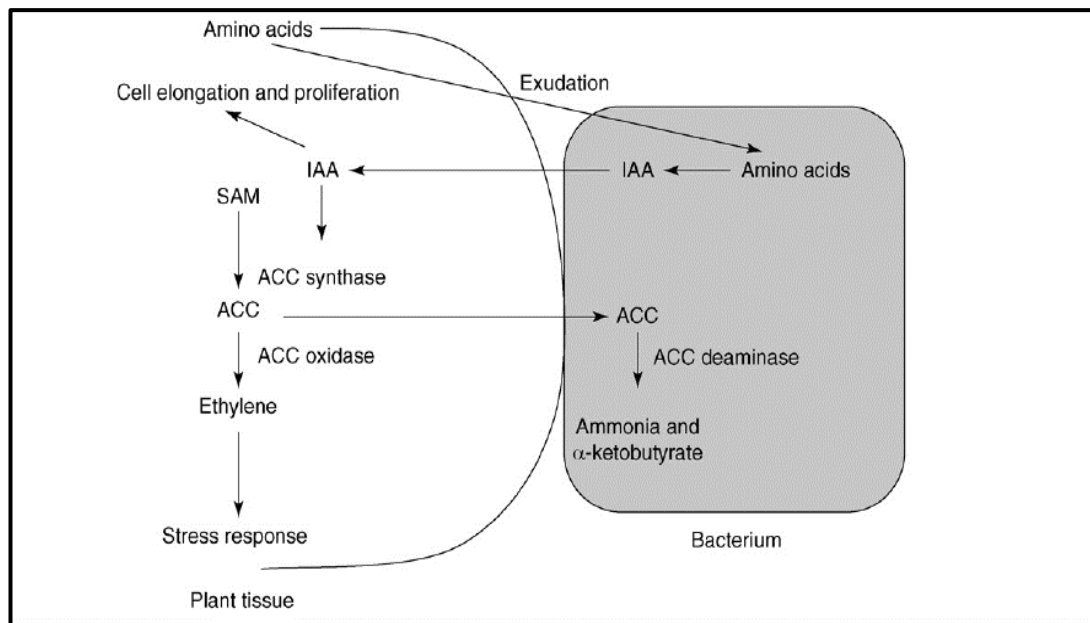


Figure 2.8: Schematic representation of how endophytic bacteria reduce the level of stress ethylene in plants.

In their study, Brigido and colleagues (2013) investigated the symbiotic performance of two *Mesorhizobium ciceri* strains transformed with an exogenous ACC deaminase gene (*acdS*) in the chickpea plant under salinity stress. One of these strains is salt-tolerant while the other is salt-sensitive. It was observed that the dry shoot weight in plant that inoculated with *acdS*-transformed salt-sensitive strain increased compared to plant that inoculated with native strain. In addition, the negative effects of salt stress on nodulation were also reduced when using *acdS*-transformed strain [Brigido et al., 2013]. A similar study was conducted by Nascimento et al. In this study, ACC deaminase producing *Mesorhizobium* strains were found to increase nodulation and promote plant growth in chickpea plant [Nascimento et al., 2012]. Another study was conducted with rhizobacteria that produce ACC deaminase. In plants inoculated with rhizobacteria producing ACC deaminase, it has been shown that ACC was hydrolyzed to ammonia and α -ketobutyrate and thereby stress ethylene level was reduced. As a consequence of that plant growth and nodulation are also promoted [Shaharoona et al., 2006].

2.4.3. Biocontrol Mechanisms

Biotic and abiotic stress factors may interfere with plant development. These stress factors may include extreme heats, high light, flood, drought, the presence of toxic metals, and environmental organic pollutants, radiation, injuries, insect predation, nematodes, high salt and various pathogens such as viruses, bacteria and fungi. In the presence of these stress factors, plant growth is less because of stress. When added to plants, endophytes can use any or several of a variety of mechanical strategies to overcome the adverse consequences of these stress conditions [Chaturvedi et al., 2016]. Plant species lacking endophytes are more susceptible to environmental stress conditions and fail to cope with pathogens [Santoyo et al., 2016]. Biocontrol mechanisms for plant growth by endophytes occurs when a bacterium restricts or prevents damage to plants that can result from a variety of pathogenic agents, including bacteria, fungi, and nematodes.

There are many mechanisms that endophytes use to promote plant growth for this purpose. Among these, there are antibiotic production, cell wall degrading enzyme synthesis, lowering of plant ethylene level, inducing of systemic resistance, reducing the amount of iron to phytopathogens in the rhizosphere, competition with detrimental microorganisms for sites on plant roots, and synthesis of pathogenic inhibiting volatile compounds (Figure 2.4).

Most endophytes isolated from plants were found to have antimicrobial activity. Antibiotics are defined as low molecular weight organic natural products produced by microorganisms, which are active even at low concentrations, against other microorganisms [Strobel, 2003]. Coronamycin which is an antibiotic with activity against human fungal pathogen *Cryptococcus neoformans* is produced by *Streptomyces* sp. isolated from *Monstera* sp. plant as an endophytic bacterium [Ezra et al., 2004]. Munumbicins A, B, C, and D are antibiotics with a wide spectrum of activity and against many plant pathogenic fungi and bacteria. These antibiotics were obtained from endophytic *Streptomyces* NRRL3052, which is isolated from *Kennedia nigricans* (Snakevine) [Castillo et al., 2002]. Another wide spectrum antibiotic that is against to especially Gr (+) bacteria is kakadumycin. It is observed that an endophytic

bacterium, *Streptomyces* sp. NRRL 30566 isolated from *Grevillea pteridifolia* produce this antibiotic [Castillo et al., 2003].

Bacterial endophytes can prevent the proliferation of plant pathogens (especially fungi) by synthesizing various antibiotics. It is interesting that some antibiotics produced by bacterial endophytes find new uses as experimental pharmaceuticals [Compant et al., 2005].

Some bacterial endophytes produce enzymes, such as cellulases, β -1,3 glucanases, proteases and lipases, which can break down part of the cell walls of many pathogenic fungi. In different studies, it has been found that the bacteria synthesize one or several of these enzymes have biocontrol activity against pathogenic fungi such as *Botrytis cinerea*, *Sclerotium rolfsii*, *Fusarium oxysporum*, *Phytophthora* spp., *Rhizoctonia solani*, and *Pythium ultimum*. Studies suggest that endophytic bacteria produce lytic enzymes such as pectinase, cellulase for host plant penetration [Lodewyckx et al., 2002].

Some bacterial species may exhibit biocontrol activity using siderophores they produce. Siderophores are active compounds that act as chelating agents for iron ions in living organisms [Glick, 2012; Ahmed and Holmström, 2014; Nair and Padmavathy, 2014]. It was mentioned earlier that bacterial siderophore formed iron-siderophore complex with ferric ion. Bacterial siderophores show a higher affinity for Fe^{+3} than the fungal siderophores. Therefore, the production of siderophore by microorganisms is beneficial to plants because it can prevent the growth of plant pathogens [Lodewyckx et al., 2002; Glick, 2012; Lacava and Azevedo, 2013; Ahmed and Holmström, 2014]. Plant growth is generally not affected from iron lacking, because most plants can grow at much lower iron concentrations than microorganisms. In addition, many plants have mechanisms to bind, take up and utilize the siderophore-iron complex formed by endophytes [Lodewyckx et al., 2002; Glick, 2012].

Although there is no precise study, competition between pathogens and endophytes may limit the incidence and severity of plant diseases. Abundant endophytic bacteria colonize plant surfaces and consume available nutrients faster than phytopathogens. This makes it difficult for pathogens to find and grow nutrients.

Plants typically respond to the presence of phytopathogens by synthesizing ethylene that exacerbates the effects of stress on plant [Abeles et al., 1992; Glick, 2012]. For this reason, one way to reduce damage to plants caused by a wide range of phytopathogens is to reduce the plant's ethylene response. Bacterial endophytes manage this by synthesizing ACC deaminase enzyme.

Bacterial endophytes can trigger a phenomenon known as induced systemic resistance (ISR) that is phenotypically similar to systemic acquired resistance (SAR), a defense mechanism that plants activate to respond to an infection caused by pathogens [Kloepper and Ryu, 2006]. Unlike SAR, ISR does not cause a visible symptom in the host plant. ISR is not directed to certain pathogens. Rather, it can be effective in controlling the diseases caused by many different pathogens. The ISR affects jasmonate and ethylene signaling in the plant and these hormones stimulate defense responses against a range of phytopathogens. It has been reported that besides ethylene and jasmonate, other bacterial molecules, such as O-antigenic side chain of bacterial outer membrane protein lipopolysaccharide, flagellar proteins, pyoverdine, chitin, β glucans, cyclic lipopeptide surfactants and salicylic acid, act as signals to stimulate systemic resistance [Compant et al., 2005; Glick, 2012]. PGPB-mediated ISR was first observed on carnation (*Dianthus caryophyllus*) plant, with reduced susceptibility to wilt disease caused by *Fusarium* sp. [van Peer et al., 1991]. In another study, it was shown that the susceptibility of cucumber plant (*Cucumis sativus*) to foliar disease caused by *Colletotrichum orbiculare* was reduced with PGPB mediated ISR [Wei et al., 1991].

Endophytic microorganisms are accepted as an effective bio-control agent, which may be an alternative to chemical control. The endophytic bacterium isolated from *Speranskia tuberculata* [Bge.] Baill., *Bacillus subtilis* was strongly antagonistic to the fungal pathogen *Botrytis cinerea*, which caused decay in tomato fruit during *in vitro* studies [Wang et al., 2009]. Another example, poplar canker is a serious disease of poplar branches that is hard to control, which is mainly caused by three fungal pathogens. In a study conducted by Ren and his colleagues (2011), it was shown that *Burkholderia pyrrocinia* JK-SH007 and *Bacillus cepacia* have strong antagonistic activity against poplar canker.

There are studies that endophytes can protect plants against parasitic nematodes and insects. According to Kloepper and his colleagues (1991), some endophytic bacteria with antagonistic effect against phytopathogenic nematodes are involved in the control of these pests. Plant damage caused by nematodes and insects emerges as a consequence of the nutritional habits and migration of these organisms and therefore limits the effect of bacterial antagonism. Thus, the control of these parasites by endophytic bacteria is more complicated and more difficult than the control of fungal and bacterial pathogens [Lodewyckx et al., 2002].

Endophytic bacteria may have multiple roles in plants. Bacterial endophytes have been found to have herbicidal activities as well as antimicrobial activity. The roles of *Bacillus* sp. SLS18, a plant growth promoting endophyte, in the biomass production and manganese/cadmium uptake of *Sorghum bicolor* L., *Phytolaccaacinososa* Roxb. and *Solanum nigrum* L. plants have been investigated. Endophytic bacteria were found to display multiple heavy metals and antibiotics resistances. The strain also exhibited the production capacity of IAA, siderophores and ACC deaminase [Luo et al., 2012].

2.5. Molecular Studies of Bacterial Endophytes

Along with the recent advances and developments in biotechnology, more studies are being carried out at the molecular level related to endophytic microorganisms, such as metagenomic studies, the use of molecular markers, molecular cloning and genetic expression studies. Molecular studies with endophytic bacteria are often based on the identification of endophytic bacteria that cannot be cultured. For example, denaturing gradient gel electrophoresis (DGGE) profiles of 16S rRNA gene fragments amplified from total plant DNA can be used to detect some non-culturable endophytic bacteria by comparing the profile with bands obtained from culturable endophytes [Araujo et al., 2002; Lacava and Azevedo, 2013; Nair and Patmavathy, 2014]. In a study conducted by Manter et al. (2010), bacterial automatic ribosomal intergenic spacer analysis (B-ARISA) and pyrosequencing were used to examine the bacterial endophytic populations of Potato (*Solanum tuberosum*) cultivar. Another method that is used to find endophytes that cannot be easily

cultivated is the metagenomic approach. Metagenomic was used to find the *acdS* operon of an uncultured endophytic microorganism, which is in interaction with *Solanum tuberosum* L. [Nikolic et al., 2011]. Molecular studies on endophytes have come to the point of sequencing the whole genome. *Enterobacter* sp. 638 is an endophytic bacterium that promotes plant growth, isolated from the poplar tree, which is a potentially important biofuel feedstock, and its entire genome has been sequenced [Taghavi et al., 2010]. Liu et al., (2012) also sequenced whole genome of an endophytic bacterium, *Enterobacter cloacae* subsp. *cloacae* strain ENHKU01.

The advanced tools of genetic engineering are highly effective in facilitating genetic manipulation of many characteristics into a single organism to achieve a defined set of goals. In this context, genetic engineering of endophytic bacteria is preferred because it is easier than genetic engineering of plants. For example, in phytoremediation studies the use of genetically modified endophytes exhibiting ACC deaminase activity with xenobiotic degradation properties may give more promising results than non-endophytes [Glick, 2004].

2.6. Endophytic Bacterial Bioremediation/Biodegradation

Bioremediation is a method used to remove contaminants and wastes from the environment by the use of microorganisms. Phytoremediation is the use of plants for extracting, degrading or stabilizing of hazardous substances in the environment. Phytoremediation emerges as a new alternative, which is economically and socially acceptable for the treatment of contaminated sites and wastewater [Lodewyckx et al., 2002]. Endophytes have the ability of breaking down complex compounds and so they are potential microorganisms to be used for bioremediation. Plant-bacterial combinations may increase the degradation of pollutants in the rhizosphere. Genetically engineered endophytic bacteria have been used to reduce the degradation caused by water-soluble, volatile or organic contaminants with phytoremediation purposes. It has been reported that metal-resistant endophytes are present in various plants growing in soil contaminated with heavy metals. Endophytic bacteria play an important role in the survival and growth of these plants. Metal-resistant endophytes promote plant growth with various mechanisms such as

nitrogen fixation, resolution of minerals and production of phytohormones and siderophores [Rajkumar et al., 2009; Rajkumar et al., 2010; Lacava and Azevedo, 2013]. Mastretta et al. (2009) isolated endophytic bacteria from surface sterilized *Nicotiana tabacum* plant seeds, grown in cadmium (Cd) and zinc (Zn) rich soils. Most of the isolated strains showed Cd tolerance. The sterilized plants were then inoculated with endophytic bacteria and it was showed that this inoculation resulted in an improved plant biomass under Cd stress conditions and higher total plant Cd concentration compared to uninoculated plants. In another study, a culture medium with an herbicide, glyphosate, as the sole carbon source, was used to isolate soybean endophytic bacterial communities. Although the cultivable endophytic bacterial community recovered from soybean (*Glycine max.*) include *Acinetobacter calcoaceticus*, *A. junii*, *Burkholderia* sp., *B. gladioli*, *Enterobacter sakazaki*, *Klebsiella pneumoniae*, *Pseudomonas oryzihabitans*, *P. straminea*, *Ralstonia pickettii*, and *Sphingomonas* sp., only *Pseudomonas oryzihabitans* and *Burkholderia gladioli* were recovered from soybean plants by glyphosate enrichment isolation. The results obtained with this study demonstrate the potential of these endophytic bacteria to be used in biodegradation programs [Kuklinsky-Sobral et al., 2005]. The genus *Methylobacterium* contains numerous endophytes which are involved in the degradation of energetic compounds, such as 2,4,6-trinitrotoluene (TNT) hexahydro-1,3,5-trinitro-1,3,5-triazine (HMX), and hexahydro-1,3,5-trinitro-1,3, triazine (RDX). It is suggested that the use of this endophytes in bioremediation and phytoremediation processes, where this type of microorganisms might be partially responsible for the degradation of environmental toxins [Lacava and Azevedo, 2013]. It is observed that the inoculation of *Brassica napus* (Canola plant) with lead (Pb) resistant endophytic bacteria, increased Pb uptake into the shoots (from 76% to 131% for *Pseudomonas fluorescens* and from 59% to 80% for *Microbacterium* sp.), when compared with the dead bacteria-inoculation control [Sheng et al., 2008]. Belimov et al. (2005) reported that bacterial *in vitro* ACC deaminase activity was positively correlated with Cd accumulation in plant tissues. Although this is a new field, these and similar studies have a lot of promise for future use of endophytic bacteria in phytoremediation studies.

3. MATERIALS AND METHODS

3.1. Plant and the Putative Endophytic Bacterium Material

Fraser photinia was “aseptically” cultured on QL [Quoirin and Lepoivre, 1977] medium containing 4.4 μM Benzyladenine (BA) as previously described [Özden-Tokatlı et al., 2008]. The putative endophytic bacterium, which was examined within the scope of this thesis, was isolated from bacterial contamination in medium and microshoots of fraser photinia *in vitro* cultures that were incubated at 25 ± 2 °C under cool white fluorescent lamps (irradiance of $36 \mu\text{mol m}^{-2} \text{s}^{-1}$) with 16h photoperiod and subcultured every 4 weeks. After 4 years of proliferation, pure bacterial contamination has occurred visually in *in vitro*-grown microshoots and medium.

3.2. Isolation of the Putative Endophytic Bacterium from Plant Material

The contaminant bacterium was taken to the sterile Tryptic Soy Broth (TSB) medium with the help of a sterile pens, along with the callus from the tissue culture sample of the plant. Bacterium was incubated at 30 °C with or without shaking and its growth examined in the TSB medium. After 72 hours, when the growth was observed, the bacterium was transferred to solid Nutrient agar (NA) and Tryptic Soy Agar (TSA) medium for pure culture control. The bacterial growth was followed for 72 h at 30 °C. Using the streak plate method, the four-step streaking was repeated three times in succession, with at least three repetitions. Since it is observed that there are no visible distinct colonies in terms of colony morphology and color, bacterial cultures are predicted to be "pure". The purified isolate stored at -80 °C and -20 °C in glycerol (v/v).

3.3. Determination of the Physiological Characteristics of the Putative Endophytic Bacterium

Physiological characteristics of the putative endophytic bacterium such as ideal growth medium, optimum growth temperature, optimum pH ranges, oxygen requirement, and motility test were identified. After determining ideal growth medium, which is Mineral Peptone Yeast Extract (MPYE), experiments were conducted with bacterium that has been grown in MPYE agar at 30 °C for 96 h.

3.3.1. Determination of the Ideal Growth Medium for the Putative Endophytic Bacterium

Contents of media that are used in growth medium determination experiments were shown in Table 3.1. All media autoclaved at 121 °C for 20 minutes, before inoculation. 15 g/L agar for microbiological media or 7 g/L agar for plant media were added for solid media.

Luria Bertani (LB) [Miller, 1972], as a rich medium and Nutrient Broth (NB), were used in the first step to grow endophytic bacterial culture which is observed in and isolated from *in vitro* tissue cultures of fraser photinia. Because the bacterial growth is rather slow on LB and NB media; MacConkey [Rodriguez-Caceres, 1982], MPYE [Davidson et al., 1989], Azotobacter medium (DSMZ, 2007) and Rhizobium Medium 1 [Atlas, 2010], and as well as the different media such as QL [Quoirin and Lepoivre, 1977], and MS [Murashige and Skoog, 1962] on which fraser photinia is cultured, were investigated for better growth of endophytic bacterium. For this purpose, pure culture of the bacteria was cultured on the different liquid and solid media mentioned. Following four-day incubation, the bacterium was found to be grown in MPYE broth and solid medium, while the bacterial growth was weak in NA and TSA media. Under these conditions, it was decided that subsequent experiments should be performed on liquid and solid MPYE medium.

Table 3.1: Contents of media that are used in growth medium determination experiments.

LB	NB	TSB	MPYE
10 g/L Trypton, 10 g/L NaCl, 5 g/L Yeast Extract pH 7.0	5 g/L Peptone from Meat, 3 g/L Meat Extract pH 7.0	17 g/L Tryptone, 3 g/L Soytone, 5 g/L NaCl, 2.5 g/L K_2HPO_4 , 2.5 g/L Glucose pH 7.3	5 g/L Yeast Extract, 3 g/L Peptone, 1.6 mM $MgCl_2$ (1 M Stock), 1 mM $CaCl_2$ (from 1M Stock) pH 7.0
Azotobacter Medium	Rhizobium Medium 1	QL	MS
5 g/L $CaCO_3$, 0,9 g/L K_2HPO_4 , 0.1 g/L $CaCl_2 \cdot 2H_2O$, 0.1 g/L KH_2PO_4 , 0,1 g/L $MgSO_4 \cdot 7H_2O$, 0.01g/L $FeSO_4 \cdot 7H_2O$, 5 g/L $Na_2Mo \cdot 2H_2O$, 5 g/L Mannitol pH 7.3	10 g/L Yeast Extract, 0.5 g/L K_2HPO_4 , 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.2 g/L NaCl, 0.002 g/L $FeCl_3 \cdot 6H_2O$ pH 7.2	1800 mg/L KNO_3 , 400 mg/L NH_4NO_3 , 356 mg/L $MgSO_4 \cdot 7H_2O$, 272 mg/L $KH_2PO_4 \cdot 2H_2O$, 1204 mg/L $Ca(NO_3) \cdot 4H_2O$, 55.6 mg/L $FeSO_4 \cdot 7H_2O$, 74 mg/L Na_2EDTA , 16.9 mg/L $MnSO_4 \cdot 4H_2O$, 0.83 mg/L KI, 6.,2 mg/L H_3BO_3 , 8.6 mg/L $ZnSO_4 \cdot 7H_2O$, 0.025 mg/L $CuSO_4 \cdot 5H_2O$, 0.25mg/L $Na_2MoO_4 \cdot 2H_2O$, 0.025 mg/L $CoCl_2 \cdot 6H_2O$ 100 mg/L Myo-Inositol, 0.5 mg/L Nicotinic Acid, 0.5 mg/L Pyridoxine.HCl, 0.1 mg/L Thiamine.HCl, 2 mg/L Glycine, 30 g/L Sucrose pH 6.0	1800 mg/L KNO_3 , 1650 mg/L NH_4NO_3 , 370 mg/L $MgSO_4 \cdot 7H_2O$, 440 mg/L $CaCl_2 \cdot 2H_2O$ 170 mg/L KH_2PO_4 , 27.8 mg/L $FeSO_4 \cdot 7H_2O$, 37.3 mg/L Na_2EDTA 22.3 mg/L $MnSO_4 \cdot 4H_2O$, 0.83 mg/L KI, 6.54 mg/L H_3BO_3 , 0.025 mg/L $CuSO_4 \cdot 5H_2O$ 0.025 mg/L $CoCl_2 \cdot 6H_2O$, 0.25mg/L $Na_2MoO_4 \cdot 2H_2O$, 8.6 mg/L $ZnSO_4 \cdot 7H_2O$, 100 mg/L Myo-Inositol, 0.5 mg/L Nicotinic Acid, 0.5 mg/L Pyridoxine.HCl, 0.1 mg/L Thiamine.HCl, 2 mg/L Glycine, 30 g/L Sucrose pH 6.0

3.3.2. Preparation of -20 and -80 °C Stock Cultures and Revitalization of the Putative Endophytic Bacterium from These Stocks

A single endophytic bacterium colony from the petri-dishes containing the endophytic bacterial culture, which was observed to be pure, was inoculated into MPYE liquid medium. Grown bacterial cultures were mixed with 80% sterile glycerol (v/v) at a ratio of 1:1 (v/v) and placed in a deep freezer, at -20 °C, for storage in sterile eppendorf tubes. For the freezer stocks of -80 °C, the bacterial culture was mixed with sterile 80% glycerol at a ratio of 1:3 (v/v) and kept in deep-freezing in sterile eppendorf tubes. In addition, the bacterial culture has been stored at +4 °C. After 1 week storage time, cultures stocked under different storage conditions were inoculated in MPYE broth for revitalization and incubated for 4 days at 30 °C.

3.3.3. Determination of the Incubation Time, the Optimum Growth Temperature, and pH of the Putative Endophytic Bacterium

Optimal growth conditions were determined by incubating the bacterium at different temperatures (22 °C, 24 °C, 30 °C, and 37 °C), different pH ranges (4.0, 4.5, 5.0, 5.5, 5.8, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0, and 10.0), and incubation periods (16-96 hours) after the selection of the medium.

MPYE, the ideal growth medium of the endophytic bacterium, was used to determine the optimum growth temperature and pH of the bacterium. Bacteria from -20 °C stock culture were inoculated on MPYE agar prepared at different pH ranges (4.0, 4.5, 5.0, 5.5, 5.8, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0, and 10.0) in order to find the optimum growth pH. At pHs below 5.8, the agar used in the medium has solidification problems. So pHs under 4 could not be examined. Bacterial growth at the relevant pH ranges was observed for 7-14 days. Incubation temperatures of 22, 30, 37 and 40 °C were chosen for determining optimum growth temperature. After inoculation of the bacterium on MPYE agar, petri-dishes placed in incubators, which are at different temperatures. Among these temperatures, 22 °C is the temperature of the plant growth chamber in which the plant is cultured. Bacterial growth at the relevant temperatures was observed for 7-14 days by OD measurements.

3.3.4. Motility Test of the Putative Endophytic Bacterium

The motility of bacterium was tested in semi-solid motility agar medium (30 g/l peptone, 3 g/L agar, 3 g/L beef extract, 0.025 g/L $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) [Atlas, 2010]. The sample was taken from four-day-old liquid culture of endophytic bacterium by a needle. The needle was stabbed 2/3 of the way down to the bottom of the tube which includes semi-solid motility agar. Thereby, a single, vertical bacterial line was formed in the medium. Following the inoculation, the cultures were incubated for 8-10 days, at 30 °C. Motile bacteria show a well dispersed growth from the line of inoculation and are considered motility positive (+), while non-motile bacteria show growth that is confined to the stab line, have sharply defined margins, and considered as motility negative (-). *Escherichia coli* was used as (+) control, while *Leuconostoc mesenteroides* was used as (-) control.

3.3.5. Determining Whether the Putative Endophytic Bacterium is a Photosynthetic Bacterium

The ability of the plant to survive without the need of subculturing in the event that the putative endophytic bacterium and the plant are co-cultured suggests that the endophytic bacterium may be a photosynthetic bacterium and may provide sugar to the plant. For this reason, it was first attempted to understand whether the bacterium was photosynthetic or not by using an anaerobic jar. The putative endophytic bacterium is aerobic. Therefore, it cannot survive under anaerobic conditions unless it produces oxygen by photosynthesis.

The putative endophytic bacterium was grown in the plant growth chamber (22 °C and 3000 lux light intensity) in Anaerop Jar (Anaerocult® 1.16387). For this purpose, the putative endophytic bacterium was inoculated on solid MPYE (pH 7) medium and incubated at 30 °C. To 9 eppendorf tubes, 900 µL of MPYE liquid medium was added. 1000 µL of MPYE liquid medium was added to another eppendorf tube. Bacterial sample was taken from a fresh culture of the putative endophytic bacterium with a sterile loop and dissolved in the eppendorf tube that contains 1000 µl MPYE liquid medium. 100 µl of bacterial suspension from this tube were diluted in each

tube to prepare 10^{-1} - 10^{-10} dilutions of the putative endophytic bacterium. In duplicate, 100 μ L of culture from each dilution was streaked on MPYE solid medium. The putative endophytic bacterial cultures were incubated in the plant growth chamber (22 °C and 3000 lux light intensity) in Anaerop Jar (Anaerocult® 1.16387) for 10-20 days until the colonies became visible.

It was also investigated whether the putative endophytic bacterium could grow in anaerobic conditions by using the BD BBL™ GasPak™ anaerobic indicator system. For this purpose, the putative endophytic bacterial culture was streaked on MPYE agar medium and petri dishes put in the gas pak according to manufacturer's manual, and incubated at 30 °C for maximum 10 days. The BD BBL™ GasPak™ anaerobic indicator is a specially designed foil envelope that contains a pad saturated with a methylene blue solution. This solution turns from blue to colorless in the absence of O₂.

3.3.6. Transmission Electron Microscopy (TEM) Analysis

TEM analysis was performed to determine the presence of photosynthetic membranes, if any, of the putative endophytic bacterium. In this procedure, bacterial culture was precipitated by centrifugation at 7000 g for 10 minutes. After centrifugation, supernatant was removed, pellet was washed with MPYE liquid medium, and the sample was centrifuged again. After removing supernatant, pellet was pre-fixed by incubating overnight (o/n) at room temperature (RT) with 0.1 M phosphate buffer (containing an equal volume of 4% (v/v) formaldehyde, 2.5% (v/v) glutaraldehyde). After pre-fixation, the pellets were washed twice with the same buffer for 15 minutes. Fixed cells were embedded in agar (3%) beads. Samples were stained at RT for 1 hour in a phosphate buffer by osmium tetroxide (OsO₄). After staining, samples were washed by same buffer thrice and dehydrated by using ethyl alcohol at different concentrations. Prepared samples were sent to Gazi University, the Faculty of Medicine for embedding in resin, cross-section and TEM analysis. Samples for imaging were photographed at 77500X magnification with TEM (LEO 906 E TEM, 80 kV, Oberkochen, Germany).

3.4. Morphological Characteristics of the Putative Endophytic Bacterium

The following tests for the determination of the morphological characteristics of the pure bacterial isolate, which is isolated from the fraser photinia tissue culture, were performed.

3.4.1. Determination of the Cell Morphology

Since there was no difference between colonies in view of morphology and color, bacterial cultures were predicted to be "pure". Samples from these colonies were stained by crystal violet and the presence of uniform colony morphology was confirmed microscopically. Then, Gram staining, which distinguishes bacteria by the chemical and physical properties of their cell walls was performed. In addition to Gram staining, lipid staining, capsule staining, and spore staining tests were also performed.

3.4.2. Determination of Colony Morphology by Light Microscope

Putative endophytic bacterium was inoculated on MPYE agar for determining colony characteristics such as colony size, shape, appearance, opacity, color, consistency, height, and margin and in MPYE broth for determining growth properties such as pellicle formation, sediment formation on the bottom, and growth on the bottom or surface. Observations were recorded after incubation at 30 °C for 5 days. At the end of the 12th day, the colonies were photographed in terms of colony size, shape, and surface properties using light microscope settings of Leica MZ16FA (Stereo Motorized Fluorescence Microscope, England) light microscope, when the colony can be clearly distinguished visually.

3.5. Determination of Colony Forming Unit (CFU)

For this purpose, the putative endophytic bacterium was inoculated on solid MPYE (pH 7) medium and incubated at 30 °C. To 9 eppendorf tubes, 900 µL of MPYE liquid medium was added. 1000 µL of MPYE liquid medium was added to another eppendorf tube. When OD₆₀₀ reached 1, bacterial sample was taken with a sterile loop and dissolved in the eppendorf tube that contains 1000 µl MPYE liquid medium. 100 µL of bacterial suspension from this tube were diluted in each tube to prepare 10⁻¹-10⁻¹⁰ dilutions of the putative endophytic bacterium. In duplicate, 100 µL of culture from each dilution was streaked on MPYE solid medium. The petri dishes were incubated at 30 °C for 10 days. After incubation the colonies on each petri dish were counted. CFU is calculated from 10⁻⁴ dilution according to the following formula:

$$CFU = \frac{\text{Number of Colony}}{\text{Dilution Factor} \times \text{Volume of Culture Plate}} \quad (3.1)$$

3.6. Biochemical Characterization

Standard biochemical tests involving oxidase, catalase, carbon utilization, oxidation/fermentation, starch, protein, lipid, and gelatin hydrolysis, nitrate reduction, indole, antibiotic susceptibility tests were performed on 96-hour-grown endophytic bacterial culture and on positive/negative control microorganisms (e.g. *Escherichia coli*, *Pseudomonas aeruginosa*).

3.6.1. Oxidase and Catalase Tests

The catalase enzyme, which generally is produced by aerobic and facultative anaerobic microorganisms, catalyzes the decomposition of hydrogen peroxide (H₂O₂) to water (H₂O) and oxygen (O₂). When added H₂O₂ to bacterial cultures grown in liquid or solid media, free O₂ can be observed as gas bubbles, indicating the decomposition of H₂O₂. This is a proof for the presence of catalase enzyme [Alexander and Strete, 2001]. For testing catalase activity, 1 ml of 3% H₂O₂ was added

to the bacterial culture in the tube or petri dish to check for occurring of oxygen bubbles. For this test, 24-h pure and fresh cultures of *Escherichia coli* [(-) control] and *Pseudomonas aeruginosa* [(+) control] were prepared by streaking on solid media.

In the oxidase test, the presence of the enzyme cytochrome C oxidase is investigated. In this system, oxygen is used as the last hydrogen receiver and the molecular oxygen is reduced to H₂O₂. This enzyme is produced by facultative anaerobes and aerobic bacteria but not by anaerobes. For this test, 24-hour pure and fresh cultures of the putative endophytic bacterium and control microorganisms (*Escherichia coli* [(-) control] and *Pseudomonas aeruginosa* [(+) control]) were prepared by streaking on solid media. N'-N'-N'-N'-tetramethyl p-phenyldiamine (1% w/v) was used as reagent. Samples of negative (*Escherichia coli*) and positive control (*Pseudomonas aeruginosa*) from 18-24 hours bacterial cultures were sampled with a sterile loop and were spread on the filter paper soaked with reagent to check if the fresh reagent was working. On the filter paper, it was observed as expected that the negative control was white and the positive control was dark purple. After proving that the reagent was working, a loop of endophytic bacterial colony was taken and spread on a filter paper soaked with reagent. The result was obtained in the first 20 seconds by comparing with controls.

3.6.2. Carbon Source Utilization Test

Carbon source utilization test in which utilization of different mono- and disaccharides and sugar alcohols, such as glucose, lactose, fructose, sucrose, mannitol, trehalose, mannose, xylose, arabinose, cellobiose and sorbitol as carbon sources was carried out on phenol red medium after 7-10 days of incubation.

Phenol Red Liquid Medium was used to perform this test on the putative endophytic bacterial isolate. This medium contains peptone and phenol red as a pH indicator. While this indicator is red in alkaline pH (> 7.0), it is yellow in acidic pH (<7.0). The Phenol Red Medium is prepared separately for each of sugars to be tested and the utilization of each sugar by the bacterium can be tested. Phenol Red Liquid medium is named according to the sugar to be tested (Phenol Red Glucose Liquid Medium etc.). When the bacterium ferments a carbohydrate, it may form acid as well

as gas as a final product. In this case, the pH decreases and the color of the red medium turn yellow. If there is a gas production at the same time, the gas accumulation is visible in the sterile Durham tubes placed inside the culture tube. If the bacterium does not use the tested sugar, the color of the medium remains unchanged [Alexander and Strete, 2001].

After preparation of the broth, 11 different sugars (glucose, fructose, cellobiose, xylose, arabinose, mannitol, sorbitol, trehalose, sucrose, mannose, lactose) were dissolved in distilled water and sterilized by passing through a 0.22 μm filter. These sugars were then added to the medium in a final volume of 0.5% (w/v). Thus, 11 liquid media with different sugar sources were prepared. Durham tube was placed in the culture tube, which includes medium with glucose, for the determination of gas formation. 100 μl of fresh 24-h culture of the bacterial isolate was inoculated in these 11 different media. The tubes were then incubated at 30 °C for 20 days. At the same time, it was observed whether or not there was gas formation in the tube which includes medium with glucose, and the results were recorded.

An additional experiment was designed to demonstrate the basicity of ambient pH after sugar use. MPYE with Bromothymol Blue (BTB-0.8% w/v) agar and Simmons' Citrate Agar (SCA) pH 7.0 media as control were prepared. Fresh culture was prepared by inoculating the endophytic bacterium into MPYE liquid medium (pH 7.0) for use in these experiments. This fresh culture was inoculated on MPYE with BTB agar medium and SCA medium with sterile loop and needle, respectively. Prepared cultures were grown at 30 °C for 4 days.

3.6.3. Oxidative/Fermentative (O/F) Test

This test is used to detect situations in which the oxidative (aerobic) or fermentative (anaerobic) metabolic pathway is used to separate the carbohydrates (especially glucose) that bacteria use. Bacteria that oxidize carbohydrates are called aerobes (A), and fermenting bacteria are called facultative anaerobes (FA). Oxidation-fermentation (O/F) medium, Hugh-Leifson medium (10% carbohydrate solution, peptone, NaCl, BTB, K_2HPO_4 and agar) is used for this test. BTB is used as a pH

indicator in the medium. In this test, the initial pH of the medium is 7.1 and the initial color of the medium is green. When pH reduces to 6.0 the color of the medium turns yellow. This medium contains low amount of peptone (required for growth). The low amount of peptone results in limited alkaline products to be formed in the medium. Thus, the neutralization of the resulting acid is prevented. The sugars are added to the O/F medium at very high concentrations. In this way the use of carbohydrates by the bacterium is promoted. Thus, the acidic products are formed in abundant amounts, the pH is reduced and the color of the medium turns yellow [Alexander and Strete, 2001].

The O/F medium was prepared as a semi-solid agar medium, and two separate tube sets. Both sets were inoculated by inserting the bacterial culture sample taken from the MPYE agar medium with sterile loop into the liquid medium. The lids of the first tube set, which inoculating process was finished, were closed. After adding sterile mineral oil which was sterilized by filtration through a 0.22 μm disposable filter to the second set of tubes, the lids were tightly closed, wrapped with parafilm, and thus anaerobic conditions were established. Both sets were incubated at 30 °C for 7-8 days. *Escherichia coli* (FA) and *Pseudomonas aeruginosa* (A) were used as control microorganisms for this test.

3.6.4. Decarboxylation Test

This test was used for the investigation of whether endophytic bacterium produce decarboxylase enzyme, which removes the carboxyl group from an amino acid. Decarboxylase broth, which contains nutrients, dextrose (a fermentable carbohydrate), pyridoxal (an enzyme cofactor for decarboxylase), and the pH indicators bromocresol purple and cresol red, was used for this test. Bromocresol purple turns purple at an alkaline pH, but at an acidic pH it turns yellow. A single amino acid was added to each broth. In decarboxylase media, three amino acids, arginine, lysine, and ornithine were tested. Each decarboxylase enzyme produced by different organisms is specific to the amino acid on which it acts. Therefore, we tested the ability of the putative endophytic bacterium to produce arginine decarboxylase,

lysine decarboxylase, and ornithine decarboxylase using three different media, which include three different amino acids.

If a microorganism cannot ferment dextrose, there will be no color change in the medium; but if it can ferment dextrose, acidic byproducts are formed; the media turns yellow because of acidity. If a microorganism may be able to decarboxylate the amino acid, alkaline byproducts such as putrescine, cadaverine are produced in the medium. Therefore, the color of the medium turns purple. If the medium is yellow, or if there is no color change, the microorganism is considered decarboxylase negative for that amino acid. If the medium turns purple, the microorganism is decarboxylase positive for that amino acid.

The putative endophytic bacterial liquid culture, were inoculated into different decarboxylase media with a final volume of 2%. After inoculation, cultures were incubated at 30 °C for 7-14 days.

3.6.5. Urease Test

Urease medium was used to determine the urease production capability of the putative endophytic bacterium. Urease is an exoenzyme that hydrolyzes urea to ammonia and CO₂. Phenol red is used in this broth as a pH indicator. Phenol red turns yellow in acidic pH ranges and fuchsia in alkaline pH ranges. If the urea in the medium is degraded and ammonia is produced, pH decreases and the color of the medium turns pink.

The putative endophytic bacterial liquid culture was inoculated in urease medium. After inoculation, cultures were incubated at 30 °C for 7-14 days. *Klebsiella pneumonia* was used as urease positive control microorganism.

3.6.6. Salt Tolerance Test

Salt tolerance test of the putative endophytic bacterium was performed using MPYE with added NaCl to create an overall salt concentration of 6.5%. Most organisms cannot survive in extreme salt concentrations. If the microorganism grows

in this medium, it may be said that this microorganism has salt tolerance. After inoculation, cultures were incubated at 30 °C for 7-14 days.

3.6.7. Hemolysis Test

Hemolysis test was performed to determine if the putative endophytic bacterium has hemolytic enzymes. For this purpose, bacterial culture was inoculated into blood agar and incubated at 30 °C for 7-14 days.

When alpha hemolysis occurs, the medium around or under the colonies is dark or greenish. When beta hemolysis occurs, the medium around or under the colonies is lightened or transparent. If the microorganism does not have hemolytic enzymes, no color change occurs in the medium. This type of hemolysis is called gamma hemolysis.

3.6.8. Hydrolytic Tests

3.6.8.1. Gelatin Hydrolysis Test (Gelatinase)

Gelatin is a protein that can be degraded by gelatinase, an extracellular enzyme. Following this enzymatic degradation, disintegration occurs in the medium contains gelatin. This disintegration is a positive (+) result indicating the presence of gelatinase. If there is no liquefaction in the medium, this is recorded as a negative (-) result [Billing, 1970].

The presence of this enzyme in the endophytic bacterium was tested with the Nutrient Gelatin (NG) medium. Bacterium sample was taken from the MPYE agar medium with the aid of a sterile needle and inoculated into NG medium to form a single vertical line. After inoculation, the bacterium was grown at 30 °C for 7-14 days. *Escherichia coli* [gelatinase (-)] and *Bacillus cereus* [gelatinase (+)] were used as control microorganisms.

3.6.8.2. Starch Hydrolysis Test (Amylase)

MPYE medium with 1% (w/v) soluble starch was prepared to test the ability of the bacterium to hydrolyze the starch, to have amylase activity, and the bacterial culture was inoculated in this medium with a sterile needle. After incubation at 30 °C for 7-14 days, 2% (w/v) Potassium iodide (KI) solution containing 1% (w/v) iodine was sprayed into the petri dishes. In this test, if the bacterium hydrolyzes the starch, light colored areas (halos) that are not stained with KI around the colonies become visible, and these strains are regarded as amylase producers [Claus, 1988].

3.6.8.3. Lipid Hydrolysis Test (Lipase)

MPYE with 1% (w/v) glycerol tributyrates was prepared to test the ability of the putative endophytic bacterium, to hydrolyze the lipids, ie, to have lipase activity. The substrate (glycerol tributyrates) in the medium was homogenized by mixing thoroughly before pouring into the petri dishes. The putative endophytic isolate was inoculated into sterilized medium and grown for 7-14 days at 30 °C. At the end of the incubation period, petri dishes analyzed under the UV light. If the halo was formed around the colony, the bacterium considered as lipase producer, lipase positive (+) [Leboffe and Pierce, 2005].

3.6.8.4. Protein Hydrolysis Test (Protease)

MPYE broth with 10% (w/v) milk powder was prepared to test the ability of the putative endophytic bacterium, to hydrolyze the proteins, ie, to have proteinase activity. The putative endophytic isolate was inoculated into sterilized medium and grown for 7-14 days at 30 °C. *Proteus vulgaris* [protease (+)] was used as a positive control. At the end of the period, if the halo was formed around the colony, the bacterium considered as protease producer, protease positive (+).

3.6.9. Nitrate Reduction Test

This test is used to determine the nitrate reduction properties of microorganisms. For this test, sterile nitrate medium was inoculated with fresh culture of the putative endophytic bacterium by using a sterile loop. Following inoculation, the tubes were tightly capped and wrapped with parafilm to allow anaerobic conditions to develop. *Pseudomonas aeruginosa* [formation of N_2 gas (+)] and *Escherichia coli* [reduction of nitrate (NO_3) to nitrite (NO_2) (+)] were used as positive controls. The bacterial cultures in the nitrate medium were incubated at 30 °C for 7-14 days. Sulfanilic acid and N, N-dimethyl-1-naphthylamine reagents were used to get results for this test. After 7-14 days of incubation, the lids of the tubes were opened, and 5 drops of each reagent were added. The tubes were agitated and left at RT for a few minutes. If red color occurs, this is a sign of nitrate reduction. If the color change does not occur within a few minutes, it is necessary to add zinc (Zn) powder to the tubes at this time and red-pink color formation is expected after 10-15 minutes [Alexander and Strete, 2001].

3.6.10. Indole Test

It was investigated whether or not endophytic bacterium produced indole by this test. Sulphite Indole Motile (SIM) medium was used for this test [Alexander and Strete, 2001]. *Escherichia coli* (indole +) was used as control microorganism and medium with no inoculation was used as negative control. Semi-solid SIM medium was inoculated in sterile conditions with putative endophytic bacterial culture by using a sterile needle. After inoculation, cultures were incubated at 30 °C for 7-14 days. Then, Kovac's Reagent was dropped into the medium as a few drops and the presence of red ring-shaped indole on the substrate was expected for positive result [Alexander and Strete, 2001].

3.6.11. Citrate Utilization Test

This test was performed to determine the ability of the putative endophytic bacterium to utilize sodium citrate as the sole source of carbon and inorganic ammonium salts as the sole source of nitrogen. For this test SCA medium was used. If the bacteria metabolize citrate, the ammonium salts are broken down to ammonia and the medium pH increases. This pH change turns the medium color from green to blue since the medium contains BTB as a pH indicator.

The putative endophytic bacterium and the positive control microorganism (*Klebsiella pneumoniae*) were inoculated into tubes which contain SCA as slant and grown at 30 °C and 37 °C, respectively.

3.6.12. Antibiotic Susceptibility Test

The antimicrobial susceptibility tests of the putative endophytic bacterial isolate was performed according to Bauer et al., (1966) by the agar diffusion method. The experiment was carried out with 20 different antibiotics belonging to the different classes: Gentamycin (10 µg), Meropenem (10 µg), Cefepime (30 µg), Ciprofloxacin (5 µg), Ampicillin (10 µg), Aztreonam (30 µg), Erythromycin(15 µg), Ampicillin-sulbactam (20 µg), Levofloxacin (5 µg), Nitrofurantoin (300 µg), Chloramphenicol (30 µg), Rifamycin (30 µg), Metsicillin (5 µg), Vancomycin (30 µg), Polymyxin B (300 U), Nalidixic Acid (30 µg), Spektinomycin (100 µg), Streptomycin (10 µg), Co-trimoxazole (25 µg), Tetracycline (30 µg). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 (Clinical and Laboratory Standards Institute, CLSI, 2013) were used as positive controls in the antibacterial effect confirmation test of the antibiotic discs used. Antibiotic discs were obtained from Bioanalysis (USA). Test results were evaluated according to the manufacturer's recommendation.

3.7. Determination of Bacterial Fatty Acid Ester Content by Gas Chromatography

Colonies of the putative endophytic bacterium were used for fatty acids and its methyl esters analysis according to the manufacturer's recommendation (Sherlock Microbial Identification System, version 6.0, MIDI, Inc., Newark, DE, USA). Fatty acid methyl esters were separated using gas chromatography (GC) (Shimadzu) with a fused-silica capillary column (25 m x 0.2 mm). The operating parameters were controlled by means of a computer program. Fatty acid methyl esters of the bacterium were identified by comparing the commercial MIS software package with the commercial database (TSBA 60).

3.8. Plant Growth Promoting Characteristics (PGPC) of the Putative Endophytic Bacterium

PGPC such as nitrogen fixation, phosphate solubilization, siderophore, IAA, and GA₃ production of the putative endophytic bacterium were investigated for the usage possibility of the bacterium as a bio fertilizer.

3.8.1. Nitrogen Fixation

Nitrogen fixation ability of the putative endophytic bacterium was analyzed by Acetylene Reduction Assay (ARA) according to Postgate [Postgate, 1978]. To determine nitrogenase activity, 8×10^6 cfu ml⁻¹ bacteria were inoculated in 9 ml Nitrogen Free Broth (NFB) (Table 3.2) in a 15 ml GC vial. A rubber stopper was used to seal the vials and vials were incubated at 30 °C. After 10 days of incubation, 1 ml acetylene gas was injected to the headspace air of the vials and the vials were incubated at 30 °C for 1 day. Reduction of acetylene to ethylene (C₂H₄) was measured with GC (Perkin Elmer Clarus 580). *Azospirillum brasilense* SP7 was utilized as positive control.

Table 3.2: Contents of NFB medium.

5 g/L	L-Malic Acid		
0,5 g/L	KH ₂ PO ₄		
0,2 g/L	MgSO ₄ .7H ₂ O		
2 ml	Microelements	0,2 g/L	Na ₂ MoO ₄ .2H ₂ O
		0,235 g/L	MnSO ₄ .H ₂ O
		0,28 g/L	H ₃ BO ₃
		0,008 g/L	CuSO ₄ .5H ₂ O
		0,024 g/L	ZnSO ₄ .7H ₂ O
1 ml	Vitamin Solution	0,01 g/L	Biotin
		0,02 g/L	Pyridoxine
4 ml	Fe-EDTA	1,64% solution	
2 ml	BTB	0,50%	
15 g/L	Agar		
pH	6.8		

3.8.2. Phosphate Solubilization

Phosphate solubilization capability of the putative endophytic bacterium was examined according to method of Sylvester-Bradley et al, (1982). Glucose-Yeast (GY) medium containing 20 g/L glucose, 10 g/L yeast extract, 20 g/L CaCO₃ was used for this purpose. Endophytic bacterium was cultivated in GY medium at 30 °C for 10 days. If the bacterium solubilized phosphorus, it is expected halos around the colonies. Colony and phosphate solubilization diameter were measured and the colony diameter was subtracted from the halo diameter. By this way solubilization halo was calculated. *Pseudomonas aeruginosa* was used as positive control for this experiment.

3.8.3. Siderophore Production

Siderophore production ability of the putative endophytic bacterium by the Chrome Azurol S (CAS) assay [Schwyn and Neilands, 1987]. For this purpose, CAS agar was prepared. Firstly, 860 ml growth medium which contains 30.24 g/L 1,4 Piperazine-diethane sulfonic acid, 1 g/L NH₄Cl, 3 g/L KH₂PO₄, 20 g/L NaCl, 9 g/L agar

was prepared and pH adjusted to 6.8 before autoclaving. Growth medium was autoclaved at 121 °C for 25 minutes and left at RT for cooling. 30 ml 10% (w/v) casaminoacids and 10 ml 20% glucose (w/v) was prepared and filter sterilized through 0.22 µm sterile filter. CAS-Fe-HDTMA was prepared at the same time. 10 ml 10 mM FeCl₃ in 100 mM HCl, 590 ml 1 mM CAS, 400 ml 2 mM HDTMA were mixed and autoclaved at 121 °C for 25 minutes. This solution was stored in a polycarbonate bottle that had been soaked o/n in 10% (v/v) HCl and rinsed for five times with ultrapure distilled water. 30 ml casaminoacids, 10 ml glucose, 100 ml CAS-Fe-HDTMA and 860 ml growth medium were mixed. Putative endophytic bacterium and positive controls, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Bacillus subtilis*, were inoculated on CAS Agar and incubated at appropriate temperatures (30 °C for endophytic bacterium and 37 °C for *Pseudomonas aeruginosa*, *Escherichia coli*, and *Bacillus subtilis*). If the microorganisms produce siderophore, orange halos on blue agar were occurred around the colonies.

3.8.4. Spectroscopic Analysis of Indole-3-Acetic Acid (IAA) Production

IAA production of putative endophytic bacterium was examined according to Sheng et al., (2008). Endophytic bacterium was inoculated in DYGS medium (0.5 g/L K₂HPO₄, 0.5 g/L MgSO₄·7H₂O, 2 g/L glucose, 1.5 g/L peptone, 2 g/L yeast extract, 1.5 g/L glutamic acid, 2 g/L malic acid, at pH 6.0) with supplemented with L tryptophan as a precursor. After 10 days of incubation at 30 °C, 1 ml cell suspension was mixed with 2 ml Salkowski's reagent and left at RT for 30 minutes. The samples were centrifuged at 10,000 g for 5 minutes and supernatant was collected. Absorbance was measured at 540 nm by UV-Visible spectrophotometer. The IAA concentration of the putative endophytic bacterium was determined using linear regression equation of standard graph.

3.8.5. Spectroscopic Analysis of GA₃ Production

The GA₃ production of the putative endophytic bacterium was investigated by spectroscopic analysis according to Holbrook with minor modifications [Holbrook et al., 1961]. For this purpose, 2 ml of endophytic bacterium culture grown in MPYE medium at 30 °C for 10 days were centrifuged at 10,000 rpm for 10 minutes. Supernatant was transferred into another clean tube and pH adjusted to 2 with 5 N HCl. Then, an equal volume of ethyl acetate was added, stirred vigorously and left for phase separation at RT. When the phase separation occurred, the supernatant was transferred into a clean tube and extraction with ethyl acetate was carried out twice as described. The whole supernatant was pooled and placed in a petri dish for the evaporation of ethyl acetate o/n at 32 °C. The following day, the extract was taken by dissolving in distilled water containing 2 ml of 0.5% Tween 80. 1 M zinc acetate containing 2 ml of 1% glacial acetic acid was added on it and left for 2 minutes at RT. 2 ml of 0.3 M potassium ferrocyanide were then added to the samples and centrifuged at 10,000 rpm for 10 minutes. Supernatant was taken and transferred into a clean tube.

The same extraction method was used for *Bacillus subtilis* and *Pseudomonas aeruginosa* cultures, which are known for GA₃ production capability. The standards of experiment were prepared to be dilutions of 5 µg/ml, 10 µg/ml, 20 µg/ml, 50 µg/ml, and 100 µg/ml using 1 mg/ml commercial GA₃ stock.

An equal volume of 30% HCl was added to the standards and all samples including controls and endophytic sample. Blank was prepared to contain 5% HCl. All samples were incubated at 20 °C for 75 minutes. The absorbance of GA₃ in the ethyl acetate phase was measured by UV spectrophotometer at 254nm. The GA₃ amount in the sample was calculated using linear regression equation of the standard curve.

3.9. Molecular Characterization of the Putative Endophytic Bacterium

Molecular identification of the putative endophytic bacterium was performed after genomic DNA and plasmid DNA isolations of the bacterium. Then, the presence of auxin, cytokinin, and virulence genes in bacterium was investigated.

3.9.1. Genomic DNA Isolation of the Putative Endophytic Bacterium

The putative endophytic bacterium was grown in MPYE broth at 30 °C for 96 hours. For bacterial genomic DNA isolation, the Promega Wizard® Genomic DNA Purification Kit (Madison, USA) was used. The genomic DNA was visualized on agarose gel (0.8%) along with 1 kb+100bp DNA ladder (Invitrogen) as size marker and quantified by UV spectrometer on 260 nm wavelengths (Shimadzu Biotech, BiospecNano Spectrometer).

3.9.2. Plasmid DNA Isolation of the Putative Endophytic Bacterium

Several different manual methods and commercial kits were used for plasmid isolation of the putative endophytic bacterium. All plasmid isolation experiments were carried out with putative endophytic bacterium, which was grown on MPYE medium at 30 °C for 10 days.

100 ml culture was pelleted by centrifugation at 13,000 rpm for 15 minutes. Supernatant was discarded and pellet was gently resuspended with 800 µl SOLI (25 mM Tris, pH 8.0; 10 mM EDTA pH 8.0; 50 mM Glucose) by pipetting up and down. After incubating at RT for 5 minutes, sample divided into 200 µl aliquots. 400 µl SOLII (0.2 N NaOH and 1% SDS) was added on the samples and mix the content by gently inverting the tube 4-5 times. Samples were incubated on ice for 5 minutes with mixing gently every 2 minutes. 300 µl SOLIII (60% 5 M Potassium Acetate (KAc) and 11,5% glacial acetic acid) was added on the samples and mix the content by gently inverting the tube 4-5 times. Samples were incubated on ice for 10 minutes with mixing gently every 2 minutes. After centrifugation at 14,000 rpm for 30 minutes (+4 °C),

supernatants were transferred into a new eppendorf tube. An equal volume of chloroform/isoamyl alcohol (24:1, v/v) was added into samples, mix thoroughly and spin at 13,000 rpm for 5 minutes in microcentrifuge. Aqueous, viscous supernatant was transferred to a fresh microcentrifuge tube, leaving the interface behind. 0.6 volumes of isopropanol were added and samples were incubated at RT for 2 hours to precipitate nucleic acids. Precipitated plasmid DNA was recovered by centrifugation at 13,000 rpm for 15 minutes. Pellet was washed with 70% ethanol twice. Ethanol was removed with care and pellet was dried in a centrifugal evaporator for 10-20 minutes. Dried plasmid DNA was dissolved in 30 μ l sterile distilled water. All samples were collected and for integrity check visualized on 1.5% agarose gel electrophoresis along with λ HindIII and 1000 bp DNA ladder as size marker. Plasmid DNA concentration was measured by UV spectrometer on 260 nm wavelength (Shimadzu Biotech, BiospecNano Spectrometer).

Plasmids DNA isolation was also performed with the DNA Spin Plasmid DNA isolation kit (Intron Bio). Plasmid DNA integrity was checked by visualizing on 1.5% agarose gel electrophoresis along with λ HindIII and 1000 bp DNA ladder as size marker. Plasmid DNA concentration was measured by UV spectrometer on 260 nm wavelength (Shimadzu Biotech, BiospecNano Spectrometer).

Although the Alkali Lysis method was tried with more than one modification, the obtained plasmid DNA was mostly degraded. The yield was low when plasmid DNA was not degraded and when plasmid DNA isolation was performed with kit. For this reason, plasmid isolation was attempted using the Macherey-Nagel Nucleospin Plasmid kit. Plasmid DNA isolation performed according to modifications suggested in the kit manual. Plasmid DNA were visualized on 1.5% agarose gel for integrity check along with λ HindIII and 1000 bp DNA ladder as size marker and plasmid DNA concentration was measured by UV spectrometer on 260 nm wavelength (Shimadzu Biotech, BiospecNano Spectrometer).

3.9.3. Auxin and Cytokinin Synthesis of the Putative Endophytic Bacterium

The presence of the *ipt* gene, which is important in cytokinin biosynthesis, and the presence of *aux1* and *aux2* genes, which are involved in auxin biosynthesis, were investigated via PCR using the genomic and plasmid DNA of the putative endophytic bacterium and degenerate primer pairs. In the amplification of the auxin genes; 5'-CTCCGATTCTTTCCAACCG-3' forward primer and 5'CGCACGTTATCCTCATACCC-3' reverse primer for *aux1* gene, 5'CTGTCAACGGAGCTGTTGGG-3' forward primer and 5'ACCCTAGTCTCATCCCAGGG-3' reverse primer for *aux2* gene [Camilleri and Jouanin, 1991] were used. In the amplification of the cytokinin gene; 5'-GAT CG (G/C) GTC CAA TG (C/T) TGT-3 ' reverse primer and 5'-GAT ATC CAT CGA TC (C/T) CTT-3' forward primer [Haas et al., 1995] were used. Ri plasmid DNA of *Rhizobacterium rhizogenes* ATCC 15834 for auxin genes and Ti plasmid DNA of *Rhizobium radiobacter* ATCC 15955 for *ipt* gene were used as positive controls. Both PCR reactions were performed in 50 µl final volume using 50-250 ng template (genomic or plasmid) DNA, 0.4 µM primer, 200 µM dNTP, 1U Taq DNA polymerase, and 2.5 µl Taq buffer. PCRs were performed as 2 min predenaturation at 94 °C, 30 cycles of 15 sec denaturation at 94 °C, 30 sec annealing at 60 °C, 1 min elongation at 72 °C, and 10 min final elongation at 72 °C steps for *aux* genes and as 2 min predenaturation at 94 °C, 30 cycles of 15 sec denaturation at 94 °C, 30 sec annealing at 55 °C, 1 min elongation at 72 °C, and 10 min final elongation at 72 °C steps for *ipt* gene. PCR reactions were performed in triplicates. After the PCR reactions, the amplified PCR products were visualised on 1.5% agarose gel along with 1 kb+100bp DNA ladder (Invitrogen) and displayed using Biorad, Universal hood II device.

3.9.4. The Gene Transfer Ability of the Putative Endophytic Bacterium to Its Host Plant

A PCR reaction for *virD1* gene amplification was performed to investigate the gene transfer ability of the putative endophytic bacterium as *Agrobacterium* Ti plasmid. For the amplification of the 440 bp *virD1* gene: 5'-ATGTCGCAAGGCAGTAGGCCACCT-3', 3'-CTACAAGGCGTCTTTCAGCAGCGAGC-5'

(Rogowsky et al., 1990) primer pairs were used. The PCR mixture was prepared with 1 µl bacterial culture as template, 5 µl Solis BioDyne 5x FIREPol Master Mix, 0.2 µM from each primer in a final volume of 25 µl. PCR programme was designed as pre-denaturation at 95 °C for 5 min and 40 cycles of denaturation at 95 °C for 1 min, primer annealing at 55 °C for 2 min and elongation at 72 °C for 2 min and final extension at 72 °C for 10 min. After the PCR reaction, the amplified PCR products were visualized on 1.5% agarose gel along with 1 kb+100bp DNA ladder (Invitrogen) and displayed using Biorad, Universal hood II device.

3.9.5. *NifH* Gene Amplification of the Putative Endophytic Bacterium

A PCR reaction for *nifH* gene amplification was performed to investigate the gene transfer ability of the putative endophytic bacterium. For the amplification of the *nifH* gene: 5'-TGCGAYCCSAARGCBGACTC-3', 3'-ATSGCCATCATYTCRCCGGA-5' degenerate primer pairs were used. The PCR mixture was prepared with 1 µl bacterial culture as template, 5 µl Solis BioDyne 5x FIREPol Master Mix, 0.2 µM from each primer in a final volume of 25 µl. PCR programme was designed as pre-denaturation at 95 °C for 5 min and 40 cycles of denaturation at 95 °C for 1 min, primer annealing at 59 °C for 2 min and elongation at 72 °C for 2 min and final extension at 72 °C for 10 min. After the PCR reaction, the amplified PCR products were visualized on 1.5% agarose gel along with 1 kb+100bp DNA ladder (Invitrogen) and displayed using Biorad, Universal hood II device.

3.9.6. Restriction Enzyme Digestion of the Putative Endophytic Bacterium Plasmid DNA

As a preliminary study of cloning studies to be carried out in the future, digesting by different restriction enzymes of plasmid DNA belonging to the putative endophytic bacterium was performed. Among these enzymes, *EcoRI*, *HpaII*, *PstI*, *MseI*, and *MspI* are Fast Digest enzymes, and enzyme digestion was performed according to the manufacturer's protocol. 1 µl of enzyme was used for 1 µg of plasmid DNA and digestion was performed at 37 °C for 5 minutes. After digestion, samples

were incubated at 80 °C for 5 minutes to inhibit the restriction enzymes. *Bam*HI digestion was performed at 37 °C for 20 minutes with 1 µg plasmid DNA and 10 U enzymes. After digestion, samples were incubated at 65 °C for 20 minutes to inhibit the restriction enzymes. *Hind*III digestion was performed at 37 °C for 30 minutes with 300 ng plasmid DNA. All restriction products were visualized on 1% agarose gel along with 1 kb+100bp DNA ladder (Invitrogen) and displayed using Biorad, Universal hood II device.

3.10. Determination of the Putative Endophytic Bacterium Effects on *in vitro* Conservation of Fraser Photinia Microshoots

In vitro cultures of fraser photinia used in this thesis were contaminant with the putative endophytic bacterium. Therefore, fraser photinia buds were taken from the garden of GTU campus in order to be used as control group and then surface sterilization followed by *in vitro* culture. Surface sterilization of buds consists of the following steps.

- The buds were washed under running tap water for at least 90 minutes.
- The shoot-tips from the washed buds were rinsed in 70% ethanol for 10-15 seconds.
- Following the ethanol wash, the samples were rinsed for 20 minutes in 1.5% Domestos.
- The samples rinsed with domestos were washed with distilled water (dH₂O).

Sterilized plant material was transferred to sterile, semi-solid QL [Quoirin and Lepoivre, 1977] medium, which include 7 g/L agar, without any plant growth regulators (QL adjusted to pH 6.0 with 1N HCl or 1N NaOH before autoclaving) for *in vitro* proliferation, and contamination rates were determined. Fraser photinia *in vitro* cultures that were incubated at 25±2 °C under cool white fluorescent lamps (irradiance of 36 µmol m² s⁻¹) with 16h photoperiod and subcultured every 4 weeks [Özden-Tokatlı et al., 2008]. In addition, shoot-tips from contaminated fraser photinia *in vitro* cultures, was 'aseptically' cultured on 4.4 µM BA containing QL medium as

described for the control group [Özden-Tokatli et al., 2008]. Shoot proliferation average number of microshoots proliferate per explant, and average length of the microshoots were evaluated after 4 weeks.

After observing the positive effects of the putative endophytic bacterium on proliferation of fraser photinia microshoots, It has been found with pre experiments that the contaminated microshoots of fraser photinia can survive at 25 ± 2 °C with 16 photoperiod under $36 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosyntetic photon flux provided by cool-white fluorescent lamps (proliferation conditions), without transferring to a fresh medium. Therefore, in order to determine the effect of the putative endophytic bacterium on the conservation of fraser photinia microshoots under *in vitro* conditions, contaminated microshoots were cultured on semi-solid sterile QL medium (medium pH was adjusted to 6.0 with 1N HCl or 1N NaOH before autoclaving), which contains $4.4 \mu\text{M}$ 6-benzyladenine (BA) and 7 g/L agar and were stored at 6, 9, 12, 15, and 16 months under proliferation conditions (25 °C, 16/8 h photoperiod), without any subculturing or transferring a fresh medium. After conservation periods, the percentage (%) of green shoots, average length (mm) of the microshoots, dry and fresh weights (g) of microshoots were calculated in order to evaluate plant quality.

In addition, leaf senescence index per microplant was calculated. For this calculation

$$\sqrt{\frac{x}{\sqrt{y + 0.5}}} \quad (3.2)$$

formula [Akdemir et al., 2010] was used. In this formula, while “x” represents the number of green leaves per microplant, “y” represents senescence plus abscised leaves per microplant. In addition, visual preference scale from 0 to 3 was also scored based on plant appearance. 0 was for dead plant; 1 was for microshoots which were brown, in some cases green; 2 was for microshoots which were green-brown; and 3 was for microshoots with bright green leaves and stems.

Moreover, influences of bacterium on shoot retrieval after conservation were also evaluated. Shoot apices were excised from *in vitro* conserved microshoots, which were contaminated, and transferred to fresh $4.4 \mu\text{M}$ BA containing QL medium in

order to assess shoot retrieval after conservation. The percentage of shoot apex that regenerated at least one elongated shoot, the average number of shoots proliferated per explant and the average length of the shoots were evaluated after 4 weeks in culture. In addition, the shoot forming capacity (SFC) index [Lambardi et al., 1993]] was also calculated based on the

$$\begin{aligned} &\text{Shoot forming capacity (SFC)} \\ &= \text{Average number of shoots per proliferating explant} \\ &\quad \times \text{Percentage of proliferating explants} / 100 \end{aligned} \quad (3.3)$$

formula.

3.11. Effects of the Putative Endophytic Bacterium on Rooting and Acclimatization

Contaminated and non-contaminated (control) microshoots with a minimum of 1-1.5 cm length, were subcultured on semi-solid QL medium which contains 4.4 μM BA, followed by microshoots were incubated with different amounts of indole butyric acid (IBA) (0.1, 0.5, and 1.0 mg/L) for rooting. After 7 days of incubation, half of the microshoots transferred to auxin free QL medium, and the remaining half was continued to be incubated on the auxin-containing medium to determine the effect of short and long-term application of auxin to rooting. Microshoots which have at least 0.2 mm root were considered as rooted. Rooting percentages of microshoots (%), number of root per microshoot, and average root length (cm) were determined after culturing for 4 weeks at $25 \pm 2^\circ\text{C}$ and $36 \mu\text{mol m}^{-2} \text{s}^{-1}$ with 16 h photoperiod. Contaminant and non-contaminant plants, which are rooted under *in vitro* conditions, were then taken from the medium, washed under running tap water, and transferred to the greenhouse conditions for acclimatization in small plastic pots containing sterile soil, peat and perlite mixture. During the first 15 days, the pots were covered with stretch film for adaptation of plants to low humidity conditions on the outside. One hole was opened on the stretch film over the day and the plants were watered at regular intervals. Fifteen days after the stretch film was completely removed, and the plants were grown in greenhouse conditions after that.

3.12. Evaluation of Data and Statistical Analysis

Biochemical and molecular analysis were repeated at least 2 times. Microshoot storage, proliferation and rooting experiments were performed by using at least 50 microshoot/explant and the experiments were repeated at least 3 times. Descriptive analysis of all study results was performed using the SPSS 12.0 package program to expeditiously see the variability in the data. The data were subjected to ANOVA to determine the differences between the experiments. When statistically different treatments were determined, the differences between the mean values were subjected to LSD multiple comparison test at $P \leq 0.05$ level. Proportional data were evaluated statistically by Chi square (X^2) test.

4. RESULTS

4.1. Determination of Physiological Characteristics of the Putative Endophytic Bacterium

Physiological characteristics of the putative endophytic bacterium such as ideal growth medium, optimum growth temperature, optimum pH ranges, oxygen requirement, and motility test were identified. After determining ideal growth medium, which is MPYE, experiments were conducted with bacterium that has been grown in MPYE agar at 30 °C for 96 h.

4.1.1. Determination of the Ideal Growth Medium for the Putative Endophytic Bacterium

As a result of experiments with different media, it was observed that the putative endophytic bacterium grown in a healthy manner on the 4th day of the incubation in MPYE liquid and solid media. Similar to the TSA and NA media used in the initial isolation studies, growth of the endophytic bacterium was weak in the Rhizobium medium and no growth in the Azotobacter medium. It was decided to use of MPYE (solid and liquid) medium which the bacterium could grow best for further experimental applications.

4.1.2. Preparation of -20 and -80 °C stock cultures and revitalization of the Putative Endophytic Bacterium from These Stocks

Bacterial stocks of -20 °C and -80 °C were prepared as described in the material method section and kept in deep freezers. Besides, bacterial culture was stored at +4 °C. After one week, these bacterial stocks were freshly cultured on MPYE liquid and solid medium. It was observed that the bacteria could grow after 4 days of incubation at 30 °C. It was concluded that long-term storage can be done in deep freezers at -20 °C and - 80 °C and the bacterium can be recovered from these stocks. It has also been observed that bacterial culture that is stored at +4 °C can revitalize.

4.1.3. Determination of the Incubation Time, the Optimum Growth Temperature, and pH of the Putative Endophytic Bacterium

Growth of the putative endophytic bacterium during the incubation period of 7-14 days was observed at the pH/temperatures presented in Table 4.1. When the results were evaluated, it was determined that at 20 °C, the visually distinguishable colonies appeared at pH 6 on the 7th day. Bacterial growth was observed beginning from day 2 at 30 °C, pH 6-7, while it was observed beginning from day 4 at pH 5.5-10. There was no bacterial growth at other temperatures at any pH range. Since no grow occur at pH 4, at temperatures of 30 °C and 20 °C, lower pH values were not tested. The results are summarized in Table 4.2.

Table 4.1: Optimum growth temperature and pH of the putative endophytic bacterium.

		pH																							
	Days	4.0		4.5		5.0		5.5		5.8		6.0		6.5		7.0		7.5		8.0		9.0		10.0	
		1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2	1	2
22°C	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Day 14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30°C	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	+/-	+/-	+/-	+/-	+/-	+/-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+z	+z	+z	+z	+?	+?	-	-
	4	-	-	-	-	-	-	+/-	+/-	+/-	+/-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	5	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+/-	+/-
	6	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+/-	+/-
	7	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Day 14	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37°C	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Day 14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
40°C	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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	6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Day 14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

4.1.4. Motility Test of the Putative Endophytic Bacterium

The putative endophytic bacterium was determined as non-motile according to the results of the experiment (Figure 4.1). The results are presented in Table 4.2.

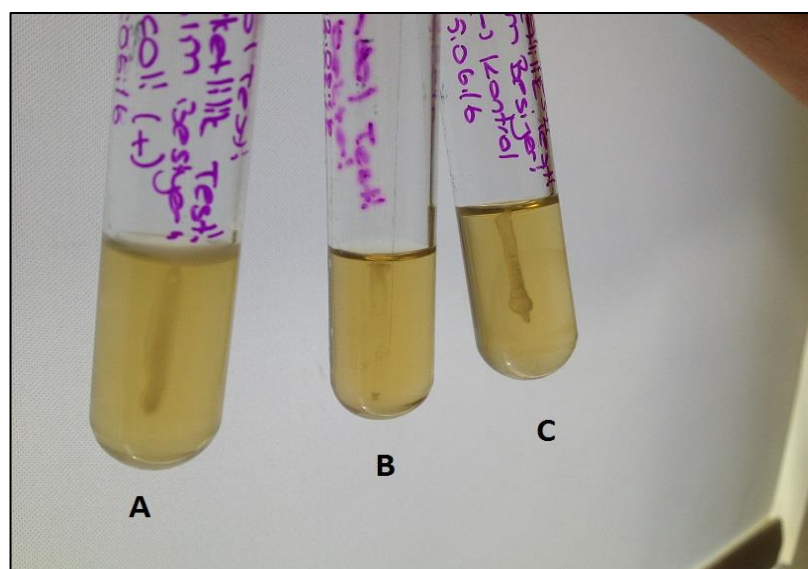


Figure 4.1: Motility test results of the putative endophytic bacterium.
A) *Escherichia coli* as (+) control, B) Endophytic bacterium, C) *Leuconostoc mesenteroides* as (-) control.

Table 4.2: Physiological characteristics of the putative endophytic bacterium.

Growth properties of the putative endophytic bacterium	
<i>Growth Properties</i>	
Aerobic growth	+ ^w
Nutrient agar, 30 °C	+
TSA, 30 °C	
MPYE, 30-34 °C	
Anaerobic growth (30-34 °C)	-
Optimum pH	7.0
Growth at pH 5.8-10.0	+
Optimum temperature	30°C
Growth at 22 and 34 °C	+
Motility	-

+^w, weak positive.

4.1.5. Determining Whether the Putative Endophytic Bacterium is a Photosynthetic Bacterium

The putative endophytic bacterium did not grow under anaerobic conditions for both experiments. It was suggested that this bacterium is not a photosynthetic bacterium. However, it was thought that this result may be due to the fact that the condition in which the bacterium was cultured was not sufficient in terms of light intensity. In this context, it was decided to investigate the presence of photosynthetic membranes in the putative endophytic bacterium by TEM analysis.

4.1.6. TEM Analysis of the Putative Endophytic Bacterium

As a result of the TEM analysis, no photosynthetic membrane structure was observed in the putative endophytic bacterium (Figure 4.2). This result supported that the putative endophytic bacterium is not a photosynthetic bacterium.

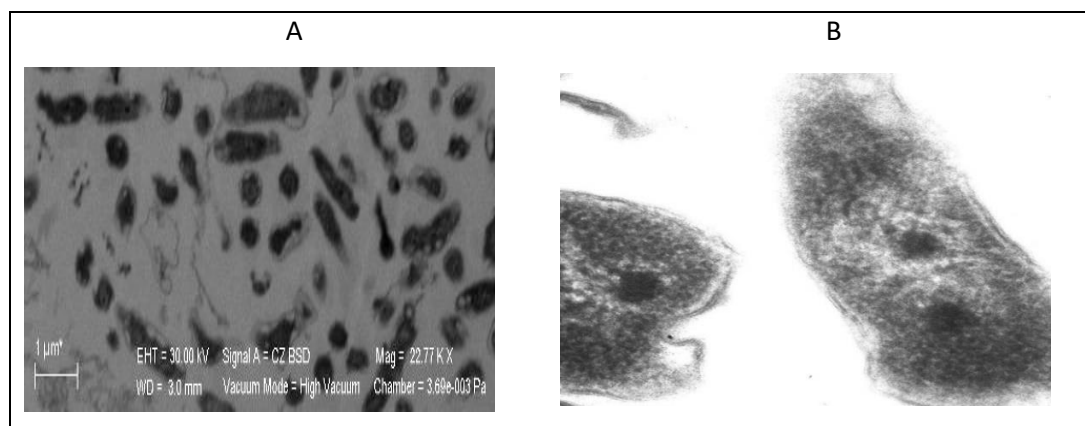


Figure 4.2: TEM photographs of the putative endophytic bacterium. A) Zeiss SEM/TEM microscopy (22700X), B) LEO 906 E (77500X).

4.2. Morphological Characteristics of the Putative Endophytic Bacterium

The determination of the morphological characteristics of the pure bacterial isolate, which is isolated from the fraser photinia tissue culture, were performed by following tests.

4.2.1. Determination of the Cell Morphology

The Gram properties [Gr (+), Gr (-)], spore condition of the isolate, β -polyhydroxybutyrate production, polyphosphate accumulation, and shape (coccus, bacillus, spiral, streptococci etc.) of the bacterial cells were microscopically determined (Table 4.3). Gram and spore staining results are showed in Figure 4.3.

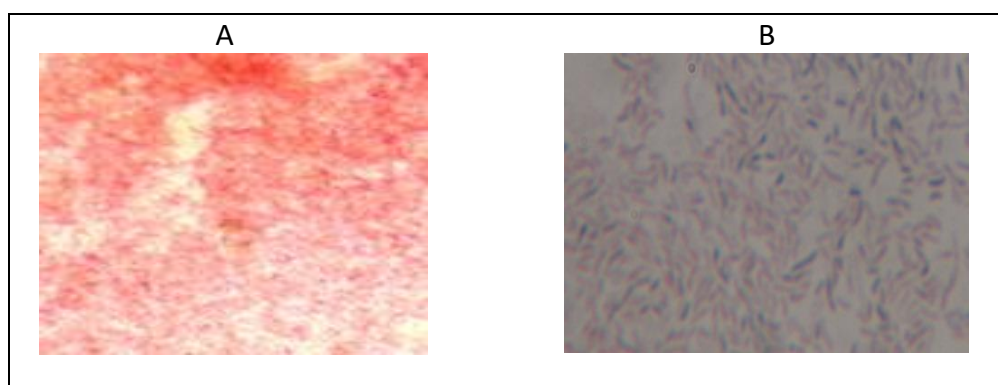


Figure 4.3: Cell morphology of the putative endophytic bacterium.
A) Gram, and B) spore staining results (1000X magnification)
of the putative endophytic bacterium.

According to these results, it was observed that the putative endophytic bacterium is gram negative, spore-free, and rod-shaped.

4.2.2. Determination of Colony Morphology by Light Microscope

It was observed that the putative endophytic bacterium grows as sediment on the bottom at the end of 5th day in MPYE liquid medium, and forms colorless, opaque, small, and high convex colonies on MPYE agar medium (Figure 4.4). At the end of the 12th day, the colonies were photographed in terms of colony size, shape, and surface properties using light microscope settings of Leica MZ16FA (Stereo Motorized Fluorescence Microscope, England) light microscope, when the colony can be clearly distinguished visually (Figure 4.4C). Results are summarized in Table 4.3.

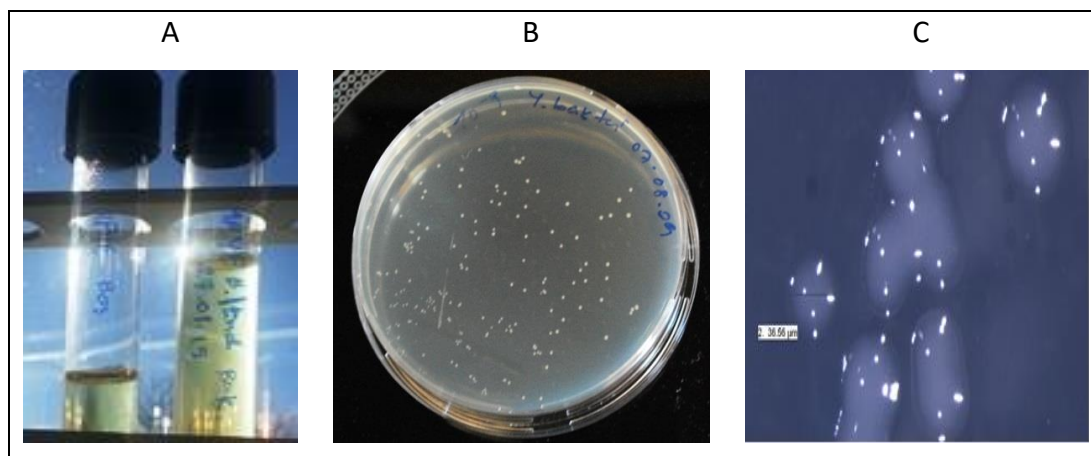


Figure 4.4: Colony morphology of the putative endophytic bacterium. A) Bacterial growth in MPYE liquid medium, B) Bacterial growth on MPYE agar medium, and C) Microscopic photograph of the putative endophytic bacterium shows colony shape and size (colony diameter: 36,56 μm).

Table 4.3: Colony characteristics of the putative endophytic bacterium.

Morphological characteristics of the putative endophytic bacterium	
Cell Morphology	
Cell Shape	Flamentous, rod
Cell height (μm)	1-3
Cell width (μm)	0.5-0.8
Gram reaction	-
Spor forming	-
Colony color	Colorless, opaque

4.3. Determination of CFU

After 10 days of incubation, the colonies on each petri dish (all dilutions) were counted. Colony numbers are represented in Table 4.4. Average colony numbers for 10^{-4} dilution was taken to calculate CFU. CFU is found as 1.7×10^7 viable cells.

Table 4.4: Colony numbers for each dilution set.

Dilutions	Colony Numbers	
	1	2
10^{-8}	0	1
10^{-7}	0	0
10^{-6}	1	1
10^{-5}	13	10
10^{-4}	164	184
10^{-3}	1400	1544
10^{-2}	WN	WN
10^{-1}	WN	WN

WN, Without Number.

4.4. Biochemical Characterization of the Putative Endophytic Bacterium

Standard biochemical tests involving oxidase, catalase, carbon utilization, oxidation/fermentation, starch, protein, lipid, and gelatin hydrolysis, nitrate reduction, indole, antibiotic susceptibility tests were performed on 96-hour-grown endophytic bacterial culture and on positive/negative control microorganisms (eg. *Escherichia coli*, *Pseudomonas aeruginosa*).

4.4.1. Oxidase and Catalase Tests

According to the results obtained, putative endophytic bacterial isolate is oxidase weak (+) (Figure 4.5) and, catalase weak (+) (Figure 4.6). Results were evaluated by comparing control microorganisms with endophytic bacterium.

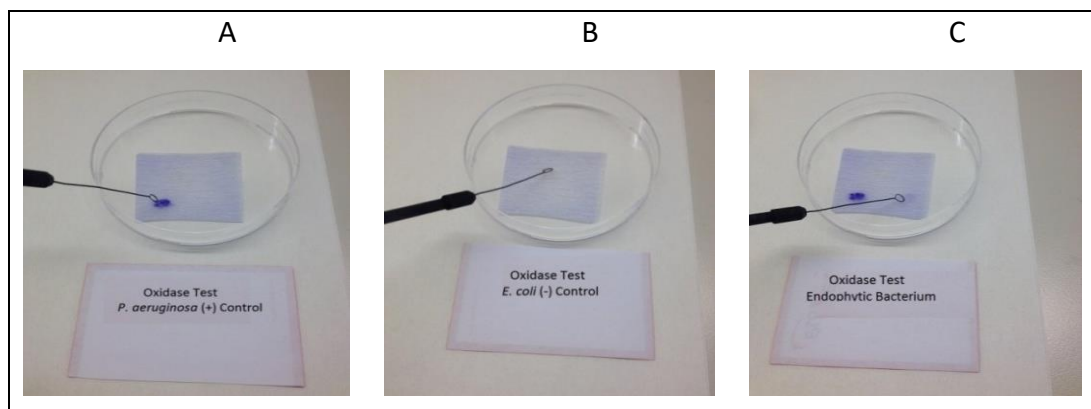


Figure 4.5: Oxidase test results of the putative endophytic bacterium. A) *P. aeruginosa* as (+) control, B) *E. coli* as (-) control, C) Endophytic bacterium.

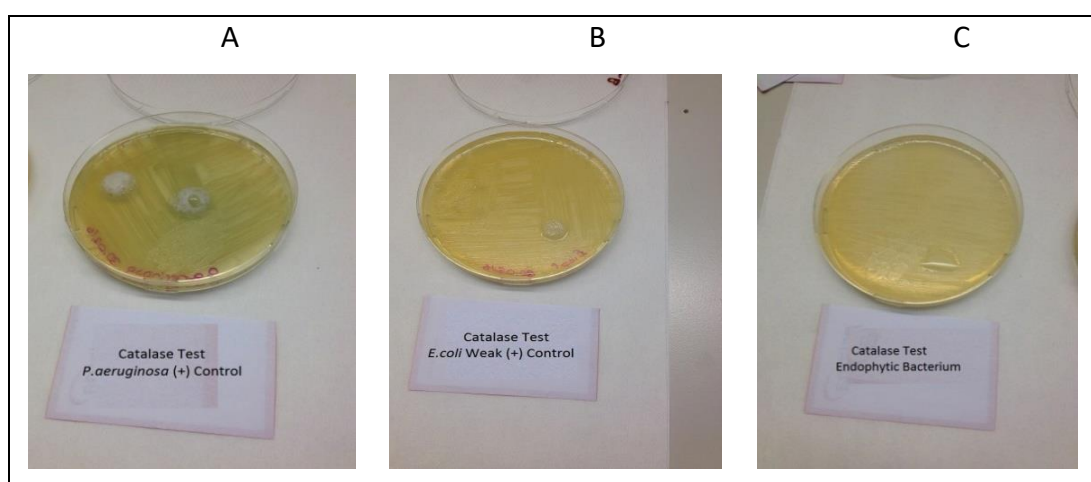


Figure 4.6: Catalase test results of the putative endophytic bacterium. A) *P. aeruginosa* as (+) control, B) *E. coli* as weak (+) control, C) Endophytic bacterium.

4.4.2. Carbon Source Utilization Test

In the carbon source utilization tests, it was found that the putative endophytic bacterium use sucrose weakly (Figure 4.7) and do not use other sugars (Table 4.5). The media which contain different sugars have not acidified despite being kept at 30 °C, for up to 20 days. On the contrary, these media have become basic with a dark red color. Despite the fact that the putative endophytic bacterium was grown, the absence of acidity in the medium led to the idea that the bacterium do not use sugars in the medium but forms ammonium using minimal levels of peptones, and create an alkaline environment. When the bacterium was cocultured with *fraser photinia*, it was emphasized that the bacterium, which produced basic byproducts, balanced the

reduced pH, which is caused by acidic by-products formed by sucrose consumption of the plant. In order to prove this opinion, the putative endophytic bacterial isolate was incubated at 30 °C for 4 days in MPYE solid medium supplemented with BTB (0.8% w/v), a pH indicator dye. The color of the medium was green at neutral pH. After 4 days of incubation the medium color has turned blue. This supported the prediction that the putative endophytic bacterium increased the pH by forming basic products, and stabilized the pH of the acidified medium in the fraser photinia tissue culture.

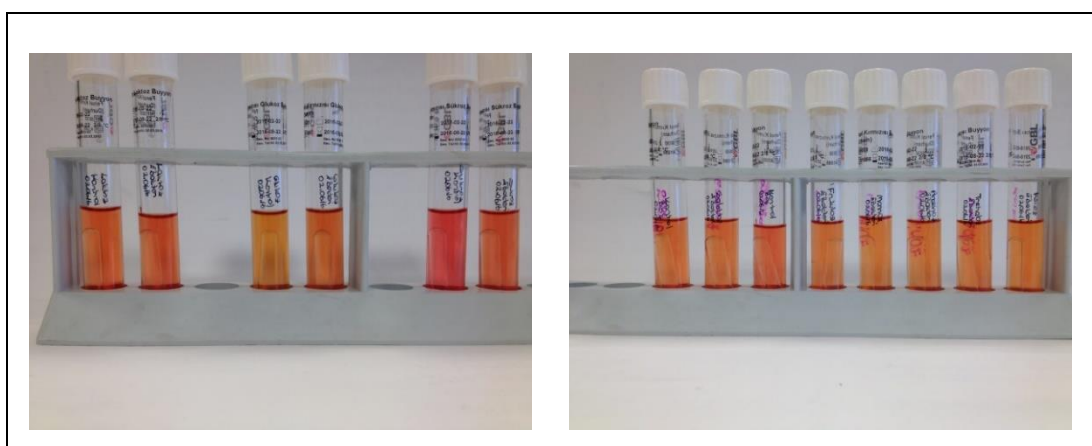


Figure 4.7: Carbon source utilization properties of the putative endophytic bacterium.

Table 4.5: Carbon source utilization properties of the putative endophytic bacterium.

<i>Carbon Source Utilization:</i>	
Glucose	-
Fructose	-
Cellobiose	-
Xylose	-
Arabinose	-
Mannitol	-
Sorbitol	-
Trehalose	-
Sucrose	+ ^w
Mannose	-
Lactose	-
<i>Gas formation:</i>	
Glucose	-

+^w, weak positive.

4.4.3. Oxidative/Fermentative (O/F) Test

As a result of this test, it was observed that the putative endophytic bacterium may be able to grow in both aerobic conditions but also the bacterium is not strictly anaerobe. So that, the bacterium is considered as facultative anaerobe (Figure 4.8).

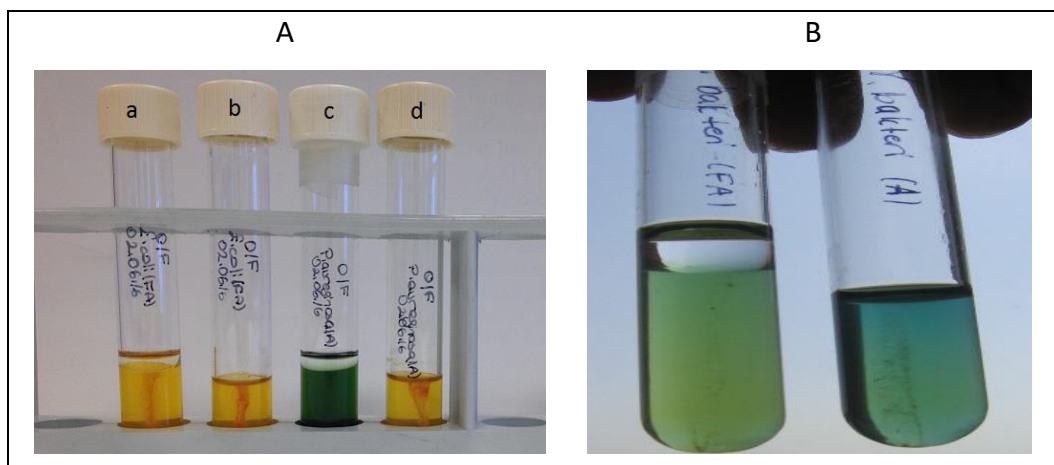


Figure 4.8: O/F test results of the putative endophytic bacterium. A) Controls, a,b) *E. coli* (FA) control, c,d) *P. aeruginosa*, B) Endophytic bacterium.

4.4.4. Decarboxylation Test

Since the putative endophytic bacterium did not use glucose, no color change from purple to yellow was observed in the media. There was no growth in the medium which contains ornithine and it was concluded that bacterium was negative in terms of ornithine decarboxylation. Besides, the putative endophytic bacterium was grown in media which contain arginine or lysine. In this context, it was considered that the putative endophytic bacterium cannot use sugar and may use lysine or arginine as the sole carbon source. But decarboxylation for these amino acids by the bacterium cannot determine (Figure 4.9).

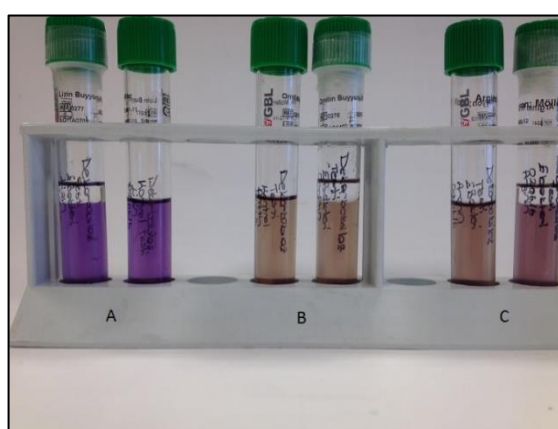


Figure 4.9: Decarboxylation test results of the putative endophytic bacterium. A) Lysine decarboxylation, B) Ornithine decarboxylation, C) Arginine decarboxylation.

4.4.5. Urease Test

As seen in Figure 4.10, the putative endophytic bacterium has no capability of urease production.

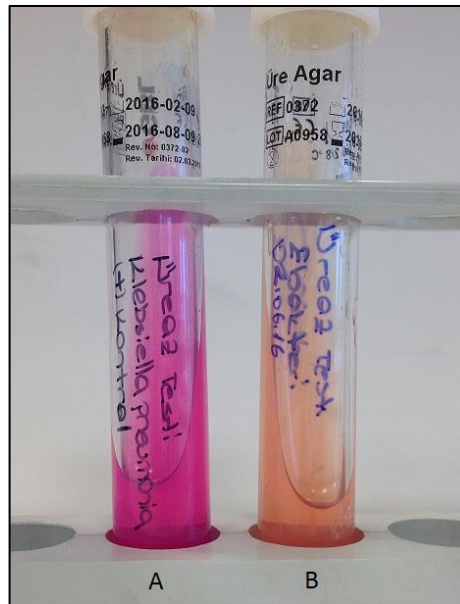


Figure 4.10: Urease test results of the putative endophytic bacterium.
A) *Klebsiella pneumoniae* (+) control, B) Endophytic bacterium.

4.4.6. Salt Tolerance Test

The putative endophytic bacterium did not grow on MPYE agar with high salt concentration. It is concluded that the putative endophytic bacterium has no salt tolerance (Data not shown).

4.4.7. Hemolysis Test

The putative endophytic bacterium was grown on blood agar. But there was no color change in the medium or under the colonies. So, the putative endophytic bacterium is considered as it has no hemolysis activity (Data not shown).

4.4.8. Gelatin, Starch, Lipid, and Protein Hydrolysis Tests

4.4.8.1. Gelatin Hydrolysis Test (Gelatinase)

As seen in Figure 4.11, the putative endophytic bacterium shows no gelatinase enzyme activity.

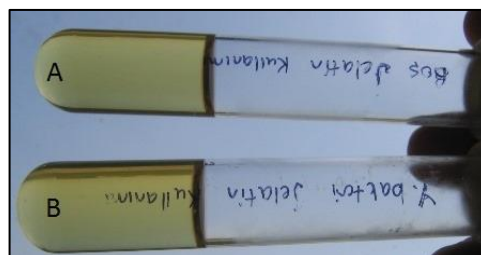


Figure 4.11: Gelatin hydrolysis test results of the putative endophytic bacterium.
A) Medium with no inoculation, B) Endophytic bacterium.

4.4.8.2. Starch Hydrolysis Test (Amylase)

After 10 days of incubation on nutrient agar medium containing soluble starch, there were no halos around the endophytic bacterium colonies. Thus, it is considered that the putative endophytic bacterium has no starch hydrolysis activity (Figure 4.12).

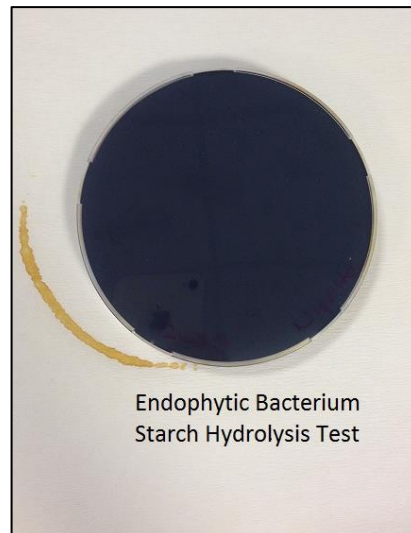


Figure 4.12: Starch Hydrolysis test result of the putative endophytic bacterium.

4.4.8.3. Lipid Hydrolysis Test (Lipase)

After 10 days of incubation on nutrient agar medium containing glycerol tributyrates, there were no halos around the endophytic bacterium colonies. Thus, it is considered that the putative endophytic bacterium has no lipid hydrolysis activity (Figure 4.13).

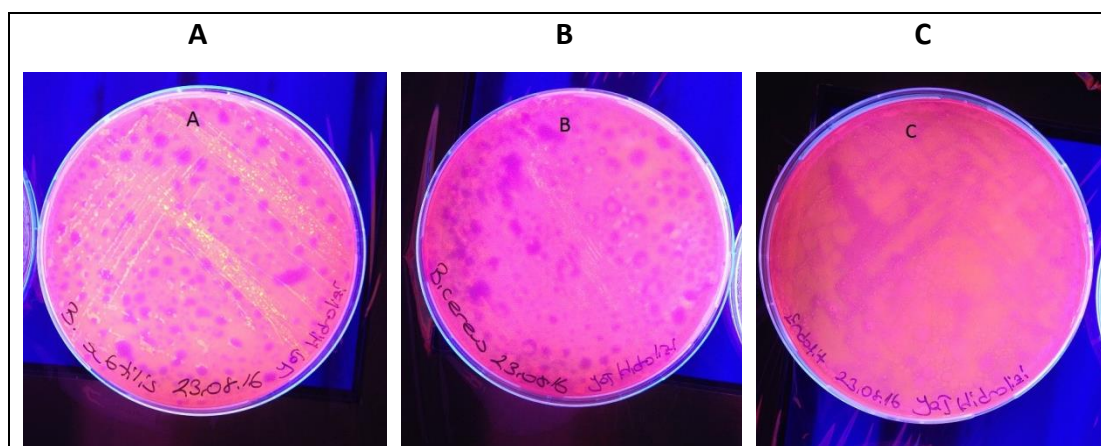


Figure 4.13: Lipid hydrolysis test results of the putative endophytic bacterium. A) *B. subtilis* (+) control, B) *B. cereus* weak (+) control, C) Endophytic bacterium.

4.4.8.4. Protein Hydrolysis test (Protease)

After 10 days of incubation on nutrient agar containing milk powder, there were no halos around the endophytic bacterium colonies. Therefore, it is considered that the putative endophytic bacterium has no protease enzyme activity (Figure 4.14).

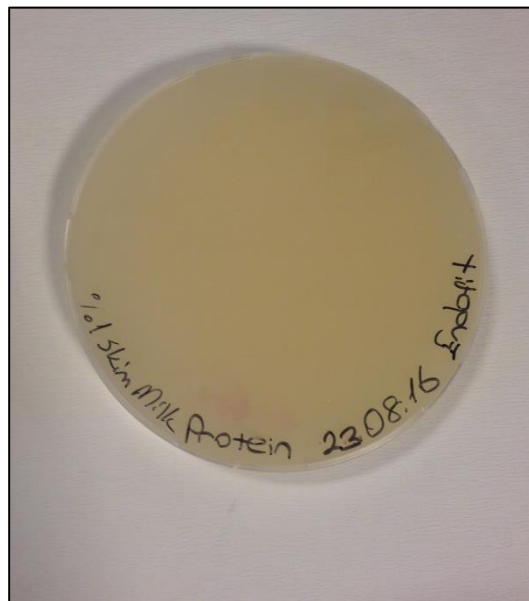


Figure 4.14: Protein hydrolysis test result of the putative endophytic bacterium.

4.4.9. Nitrate Reduction Test

As seen Figure 4.15A, N_2 gas accumulation occurs in all Durham tubes, although it is weak in the putative endophytic bacterium. Medium color has changed into red in *E. coli* sample as NO_3 was reduced to NO_2 . No color change has occurred in *P. aeruginosa* sample because it has reduced nitrate to N_2 gas (Figure 4.15B). Since the color change did not occur in the first few minutes, zinc powder was added into tubes containing the putative endophytic bacterium. After adding zinc powder, medium color turned pink (Figure 4.15C). It is shown that the putative endophytic bacterium may reduce NO_3 to NO_2 and also to N_2 gas.

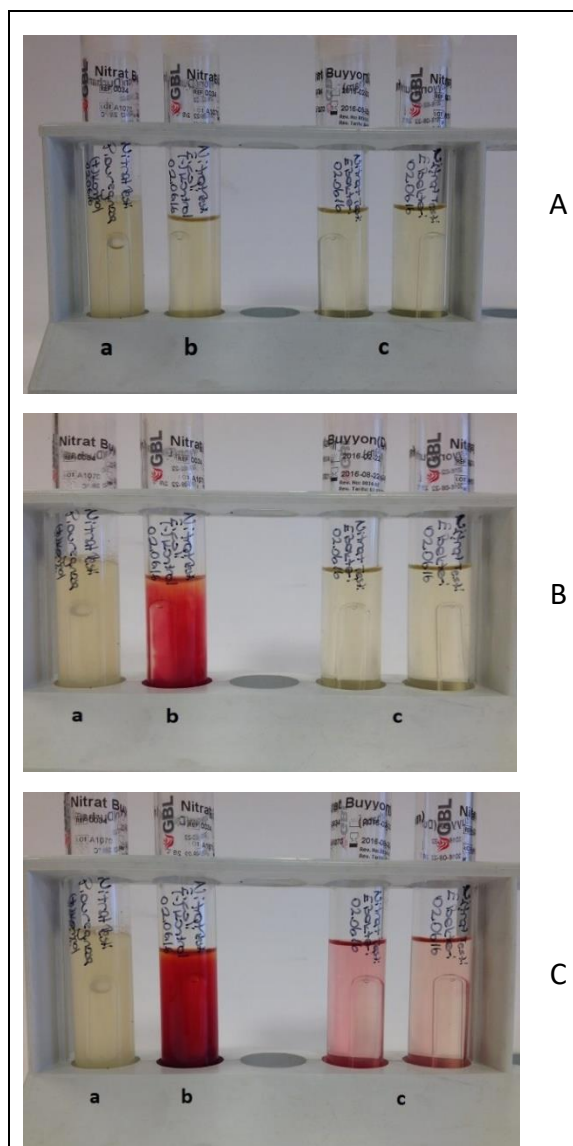


Figure 4.15: Nitrate reduction test results of the putative endophytic bacterium.
a) *P. aeruginosa*, b) *E. coli*, and c) Endophytic bacterium.

4.4.10. Indole Test

As seen in Figure 4.16, the putative endophytic bacterium does not produce indole.

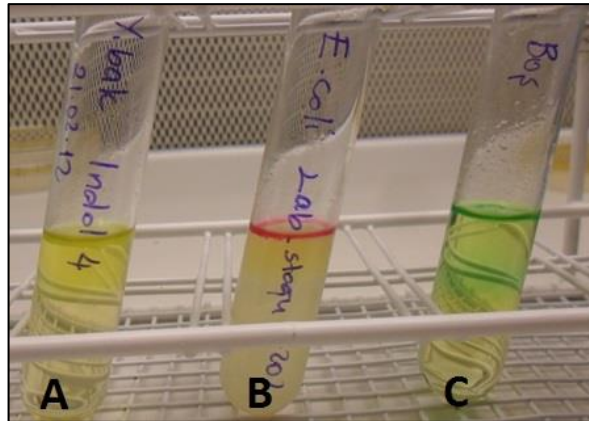


Figure 4.16: Indole test result of the putative endophytic bacterium. A) Endophytic bacterium, B) *E.coli* as (+) control, and C) Medium with no inoculation.

4.4.11. Citrate Utilization Test

As seen in Figure 4.17, the putative endophytic bacterium cannot metabolize citrate. The results of the overall biochemical tests were summarized in Table 4.6.

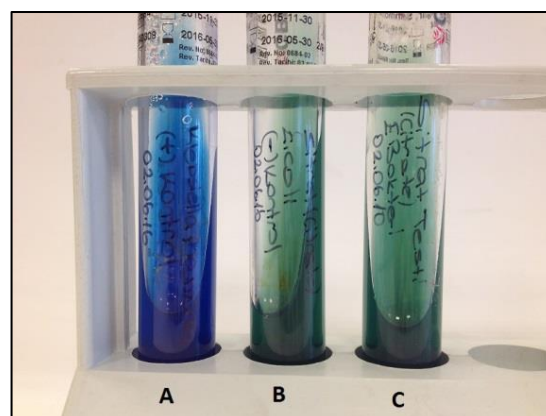


Figure 4.17: Citrate test results of the putative endophytic bacterium. A) *Klebsiella pneumoniae* as (+) control, B) *E. coli* as (-) control, and C) Endophytic bacterium.

Table 4.6: A summary of biochemical characteristics of the putative endophytic bacterium.

Catalase	+ ^w
Oxidase	+ ^w
Oxidative/Fermentative	FA
Starch hydrolysis	-
Gelatin hydrolysis	-
Protein hydrolysis	-
Lipid hydrolysis	-
Lysine decarboxylation	-
Arginine decarboxylation	-
Ornithine decarboxylation	-
Urease	-
Salt tolerance	-
Hemolysis	-
Nitrate reduction:	
$\text{NO}_3 \rightarrow \text{NO}_2$	+
$\text{NO}_2 \rightarrow \text{N}_2$ (gas formation)	+
Indole production	-
Citrate metabolization	-

+^w, weak positive reaction; FA, Facultative anaerobic; +, positive reaction; -, negative reaction.

4.4.12. Antibiotic Susceptibility Test

According to the results obtained, the putative endophytic bacterium is sensitive to Streptomycin (10 µg), Tetracycline (30 µg), Nalidixic Acid (30 µg), Polymyxin B (300 U), Spektrimycin (100 µg), and Erythromycin (15 µg) antibiotics. However, it is resistant to all other tested antibiotics. The results are represented in Table 4.7.

Table 4.7: Antibiotic susceptibility test results of the putative endophytic bacterium.

<i>Antibiotic Susceptibility</i>	
Gentamycin (10 µg)	R
Meropenem (10 µg)	R
Cefepime (30 µg)	R
Ciprofloxacin (5 µg)	R
Ampicillin (10 µg)	R
Aztreonam (30 µg)	R
Erythromycin (15µg)	S
Ampicillin-sulbactam (20 µg)	R
Levofloxacin (5 µg)	R
Nitrofurantoin (300 µg)	R
Chloramphenicol (30 µg)	R
Rifamycin (30 µg)	R
Meticillin (5 µg)	R
Polymyxin B (300 U)	S
Vancomycin (30 µg)	R
Nalidixic acid (30 µg)	S
Spectinomycin (100 µg)	S
Streptomycin (10 µg)	S
Co-trimoxazole (25 µg)	R
Tetracycline (30 µg)	S

S, sensitive; R, resistant.

4.5. Determination of Bacterial Fatty Acid Ester Content by Gas Chromatography

As a result of determination the fatty acid methyl esters of the putative endophytic bacterium by GC, the highest fatty acids are found to be C18:1W7c (77.28%) and C16:0 (6.61%), respectively. It was also determined that C18:0 (4.61%), C17:0 iso (3.68%), C19: 0 CycloH8c (3.57%), C17:0 (2.62%) and C18:1w9c (1.63%) were also present in small amounts (Figure 4.18). When the results are evaluated

according to the TSBA 60 database, it was found that the putative endophytic bacterium was similar to *Ochrobactrum anthropi* (Achromobacter Vd, CDC group Vd, 0,642) and *Phyllobacterium myrsinacearum* (0,417).

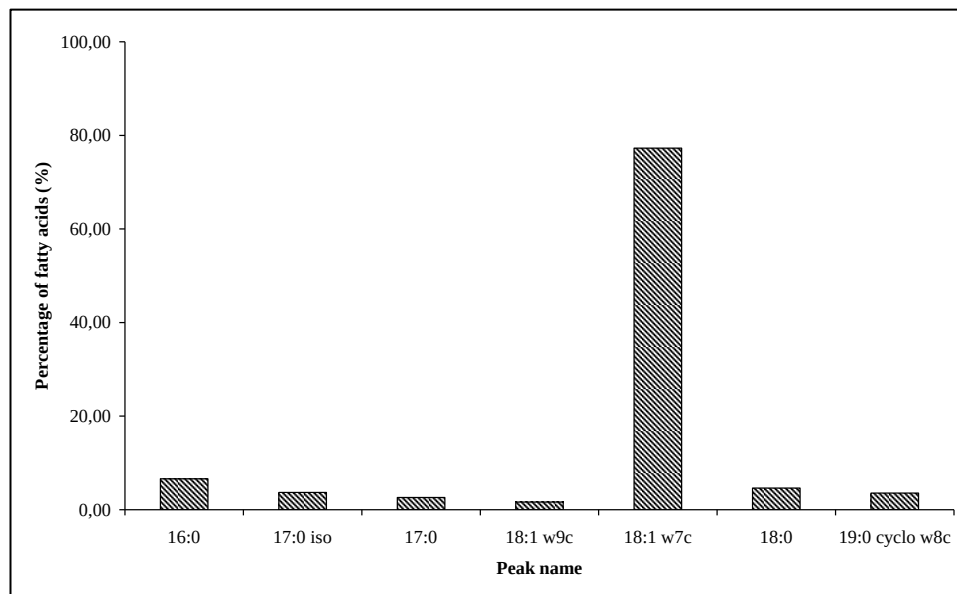


Figure 4.18: FAME analysis of the putative endophytic bacterium.

4.6. Plant Growth Promoting Characteristics (PGPC) of the Putative Endophytic Bacterium

PGPC such as nitrogen fixation, phosphate solubilization, and siderophore, IAA, and GA₃ production of the putative endophytic bacterium were investigated.

4.6.1. Nitrogen Fixation

The putative endophytic bacterium has nitrogen fixation ability according to results that obtained by ARA. That means the bacterium has also nitrogenase activity (Figure 4.19).

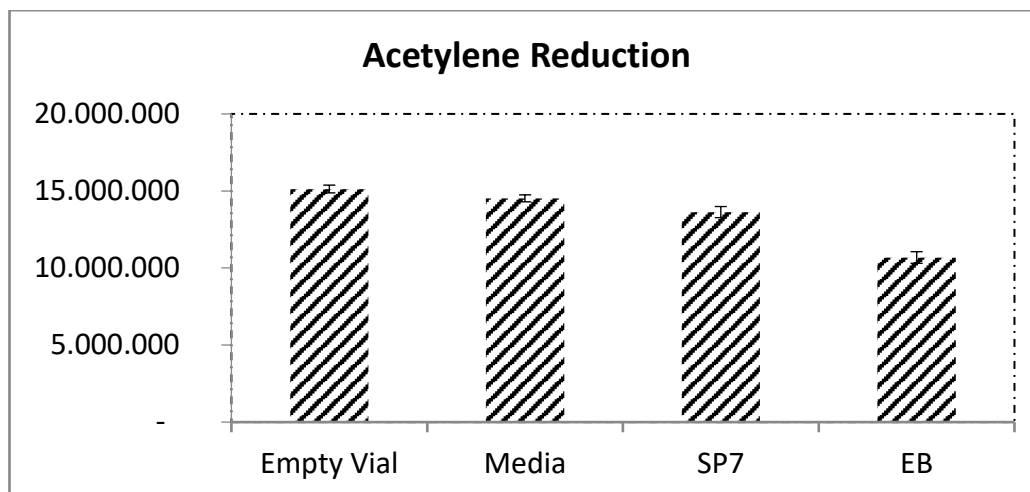


Figure 4.19: Nitrogen fixation results of the putative endophytic bacterium.

4.6.2. Phosphate Solubilization

It was observed that the putative endophytic bacterium cannot solubilize phosphorus (Data not shown).

4.6.3. Siderophore Production

After 10 days of incubation, it was observed that the putative endophytic bacterium does not produce siderophore (Figure 4.20).

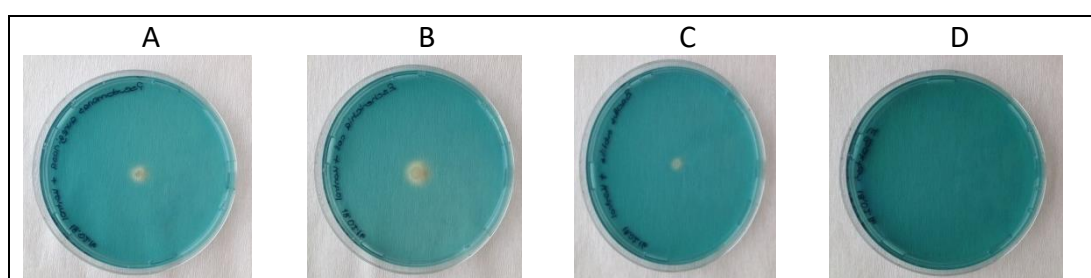


Figure 4.20: Siderophore production test result of the putative endophytic bacterium. A) *P. aeruginosa*, B) *E. coli*, C) *B. subtilis*, D) Endophytic bacterium.

4.6.4. Spectroscopic Analysis of IAA Production

According to test results it was observed that the putative endophytic bacterium produce IAA at $10\ \mu\text{g ml}^{-1}$ concentration (Figure 4.21).

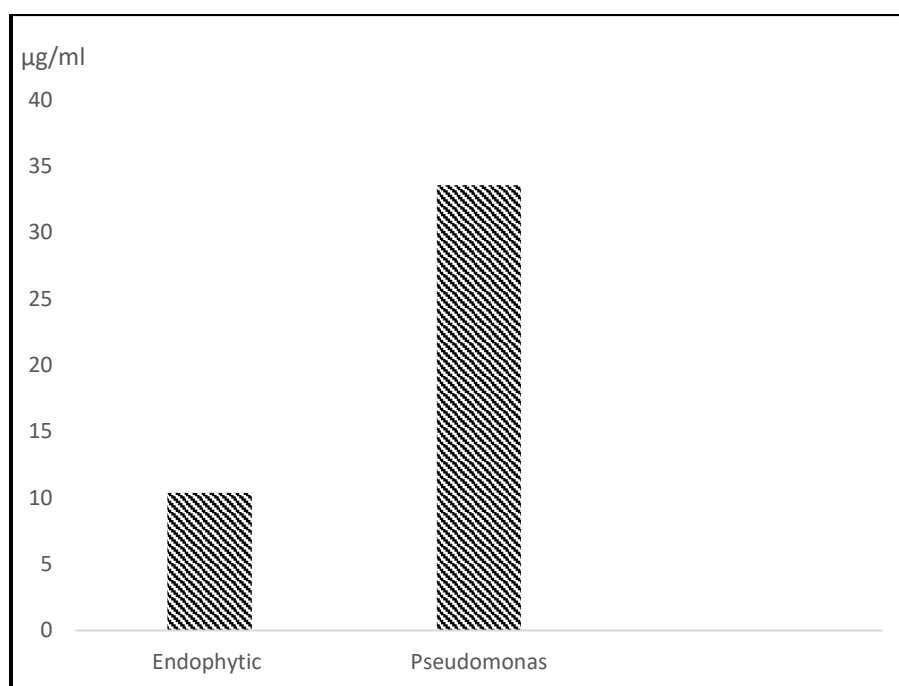


Figure 4.21: IAA production of the putative endophytic bacterium.

4.6.5. Spectroscopic Analysis of GA₃ Production

As seen in Figure 4.22, the putative endophytic bacterium produces GA₃ at $29\ \mu\text{g ml}^{-1}$ concentration.

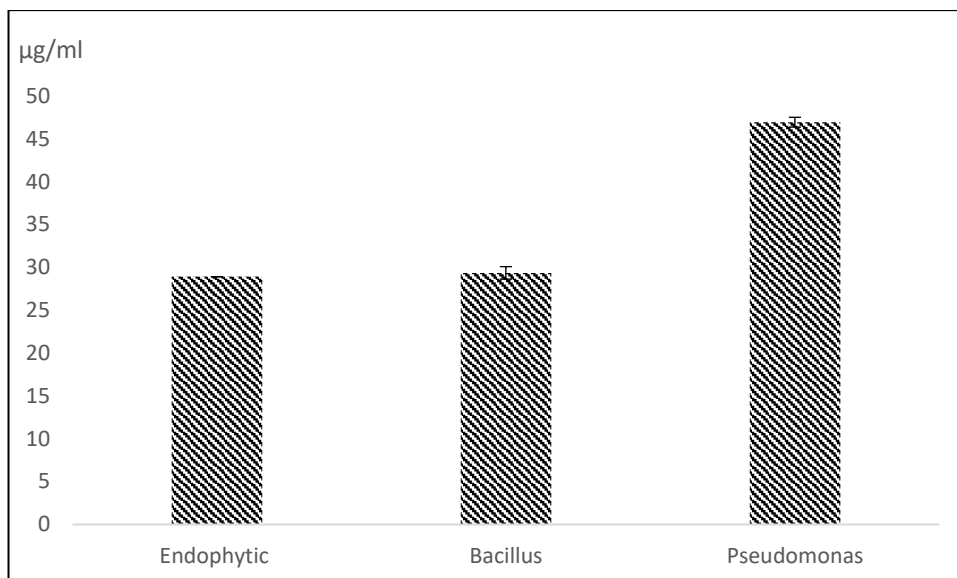


Figure 4.22: GA₃ production of the putative endophytic bacterium.

4.7. Molecular Characterization of the Putative Endophytic Bacterium

Molecular identification of the putative endophytic bacterium was performed after genomic DNA and plasmid DNA isolations of the bacterium. Then, the presence of auxin, cytokinin, and virulence genes in bacterium was investigated.

4.7.1. Auxin and Cytokinin Synthesis of the Putative Endophytic Bacterium

When PCR with *aux1* and *ipt* primers was performed using genomic DNA as a template, no band was obtained. When PCR with *aux1* primers was performed using plasmid DNA as template, ~950 bp and 791 bp single bands were obtained for endophytic bacterium and *Rhizobacterium rhizogenes* ATCC 15834 (Figure 4.23). When PCR with *ipt* primers was performed using plasmid DNA as template, ~800 bp and 427 bp single bands were obtained for endophytic bacterium and *Rhizobacterium radiobacter* ATCC 15955 (Figure 4.23). The PCR results show that *aux1* and *ipt* genes are present in the putative endophytic bacterium, albeit in different sizes than control microorganisms. When PCR with *aux2* primers was performed using plasmid

DNA as template, multiple bands were obtained for both endophytic bacterium and *Rhizobium rhizogenes* ATCC 15834 (Data not shown).

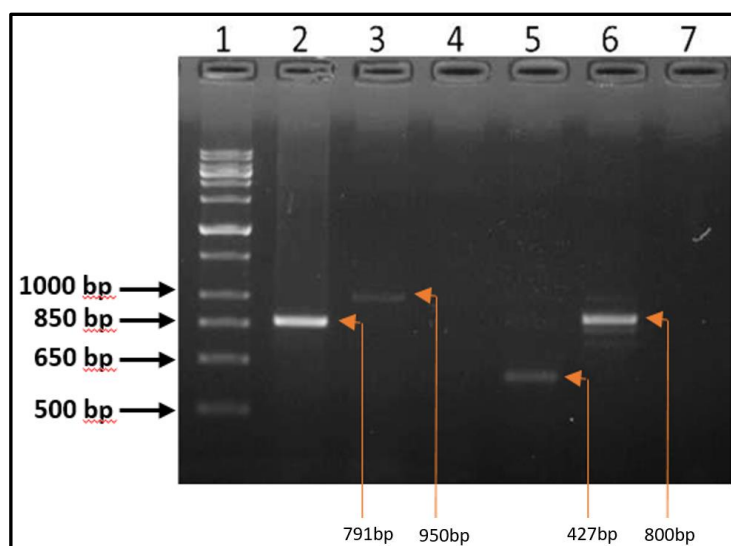


Figure 4.23: Agarose gel electrophoresis of PCR amplification products obtained with *aux1* and *ipt* gene primers by using plasmid DNA as template. 1. 1000 bp size marker (250 bp, 500 bp, 750 bp, 1000 bp, 1500 bp, 2000 bp etc.), 2. PCR amplification of *Rhizobacterium rhizogenes* ATCC 15834 plasmid DNA (pRi) with *aux1* primers, 3. PCR amplification of endophytic bacterium plasmid DNA with *aux1* primers, 4. PCR amplification with *aux1* primers as negative control, 5. PCR amplification of *Rhizobacterium radiobacter* ATCC 15955 plasmid DNA (pTi) with *ipt* primers, 6. PCR amplification of endophytic bacterium plasmid DNA with *ipt* primers, and 7. PCR amplification with *ipt* primers as negative control.

4.7.2. The Gene Transfer Ability of the Putative Endophytic Bacterium

When PCR with *virD1* primers was performed using bacterial culture as template, ~550 bp single band was obtained for endophytic bacterium (Figure 4.24). The PCR results show that *virD1* gene is present in the putative endophytic bacterium.

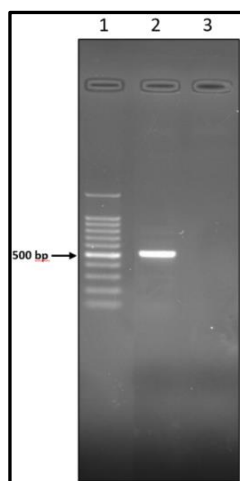


Figure 4.24: Agarose gel electrophoresis of the PCR amplification *virD1* genes by using endophytic bacterial culture. 1. 100 bp marker (100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, 1000 bp, 1500 bp), 2. Bacterial *virD1* gene amplification, 3. PCR amplification with *virD1* primers as negative control.

4.7.3. *NifH* Gene Amplification of the Putative Endophytic Bacterium

When PCR with *NifH* primers was performed using bacterial culture as template, ~750 bp single band was obtained for endophytic bacterium (Figure 4.25). The PCR results show that *NifH* gene is present in the putative endophytic bacterium.

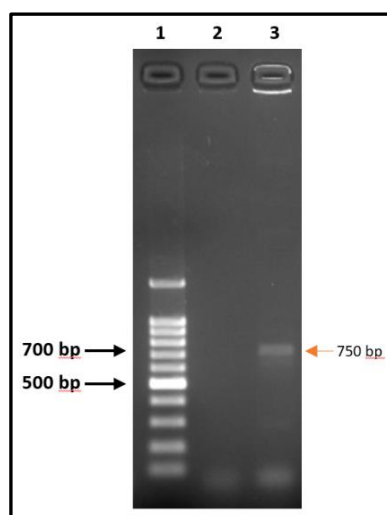


Figure 4.25: Agarose gel electrophoresis of the PCR amplification *nifH* genes by using endophytic bacterial culture. 1. 100 bp marker (100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, 1000 bp, 1500 bp), 2. PCR amplification with *nifH* primers as negative control, 3. Bacterial *nifH* gene amplification.

4.7.4. Restriction Enzyme Digestion of the Putative Endophytic Bacterium

The isolated plasmid DNA was digested by various restriction enzymes previously mentioned in the Materials and Methods section. When the plasmid DNA is digested with *Pst*I, *Msp*I, *Mse*I and *Hpa*II, the DNA is completely degraded. It was observed that the *Eco*RI, *Bam*HI and *Hind*III enzymes does not digest the plasmid DNA (Figure 4.26).

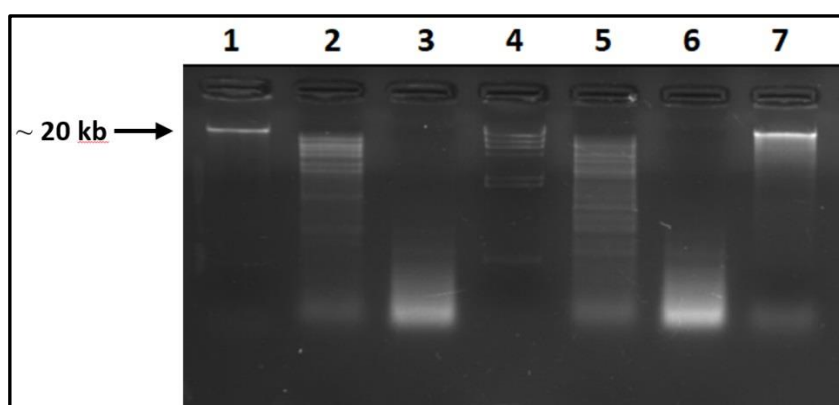


Figure 4.26: Agarose gel electrophoresis of the digested plasmid DNA with various restriction enzymes. 1. Uncut plasmid DNA, 2. *Pst*I, 3. *Msp*I, 4. λ /*Hind*III Marker, 5. *Mse*I, 6. *Hpa*II, and 7. *Bam*HI.

4.8. Determination of the Putative Endophytic Bacterium Effects on *in vitro* Conservation of Fraser Photinia Microshoots

Shoot apices of fraser photinia which were taken from GTU and sterilized to be used as control, were cultured at 25 ± 2 °C with 16 h photoperiod under $36 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity. After 4 weeks, 40% contamination was observed in shoot apices of fraser photinia. 38.8% of the noncontaminated shoot apices were proliferated. 1.8 microshoots per explant obtained in noncontaminated shoot apices and average length of these microshoots were 5.5 mm (Table 4.8, Figure 4.27). 100% of the contaminated shoot apices with our putative endophytic bacterium were proliferated and 3.8 microshoots per explant were obtained (Table 4.8). The average length of these microshoots was about 12 mm. The relatively high proliferation of shoot apices containing endophytic bacterium as contaminant than control group

may be due to culturing in the medium with cytokinin that promotes proliferation of the shoot apices or may be caused by the putative endophytic bacterium. Therefore, the shoot apices from control group and contaminated microshoots were cultured on QL or QL with 4.4 μ M BA (Table 4.9). After 4 weeks of culture, proliferation rates of the shoot apices were compared with proliferation rates of the shoot apices from microshoots, which contain contaminant.

Table 4.8: *In vitro* proliferation of shoot apices from fraser photinia which contains endophytic bacterium or not.

<i>In vitro</i> cultures	Proliferation ^b (%)	Microshoot/explant ^{cd}	Microshoot length ^{cd} (mm)
Control ^e	38.8b	1.80 $\pm$ 0.16b	5.50 $\pm$ 0.04b
Contaminated ^e	100a	3.87 $\pm$ 0.12a	11.96 $\pm$ 0.24a

^a The data were collected 30 days after culture initiation. Each trial was made with at least 50 explants and the trials were repeated at least twice.

^b The same letters following the percentages show no statistical difference compared to the Post Hoc Multiple comparison test ($P \leq 0.05$).

^c Standard error of the mean $\pm$.

^d The same letters following the averages show no statistical difference compared to the LSD test following ANOVA ($P \leq 0.05$; The average difference was analyzed horizontally).

^e Shoot apices from control samples were cultured on OL medium with plant growth regulator; shoot apices from contaminated samples were cultured on QL medium with 4.4 μ M BA.

^f Contaminated with our putative endophytic bacterium.



Figure 4.27: *In vitro* proliferated shoot apices of fraser photinia.

Table 4.9: After first subculture, *in vitro* proliferation of shoot apices from control and contaminated microshoots on media which contain plant growth regulator (OL+4.4µM BA) or not (QL0).

<i>In vitro</i> cultures	Proliferation ^b (%)	Microshoot/explant ^{cd}	Microshoot length ^{cd} (mm)
QL0			
Control	92ab	1.00±0.00c	4.00±0.51b
Contaminated	80b	1.00±0.00c	3.27±0.53b
QL+4.4 µM BA			
Control	100a	2.20±0.10b	4.45±0.33b
Contaminated	100a	3.80±0.10a	10.90±0.50a

^a The data were collected 30 days after culture initiation. Each trial was made with at least 50 explants and the trials were repeated at least twice.

^b The same letters following the percentages show no statistical difference compared to the Post Hoc Multiple comparison test ($P \leq 0.05$).

^c Standard error of the mean $\pm$.

^d The same letters following the averages show no statistical difference compared to the LSD test following ANOVA ($P \leq 0.05$; The average difference was analyzed horizontally).

According to the results obtained from the 4 weeks culture, control group and contaminated shoot apices in the medium containing 4.4 µM BA showed 100% proliferation (Table 4.9). In the medium which has not contained cytokinin, although the shoot apices from control group showed more proliferation, there was no statistically difference compared to the shoot apices from contaminated group. Maximum microshoots number per explant and lengthiest microshoots were obtained by culturing the contaminated shoot apices on cytokinin contained QL. This data demonstrates that the putative endophytic bacterium has no detrimental effect on *in vitro* proliferation of the plant cultured in QL medium without cytokinin. In the medium containing cytokinin, it was determined that this contamination increases the multiple shoot forming capacity of the shoot apices.

After observing the positive effects of the putative endophytic bacterium on proliferation of fraser photinia microshoots, It has been found with pre experiments that the contaminated microshoots of fraser photinia can survive at 25 ± 2 °C with 16 photoperiod under $36 \mu\text{mol m}^{-2} \text{s}^{-1}$ light provided by cool-white fluorescent lamps

(proliferation conditions), without transferring to a fresh medium. Therefore, in order to determine the effect of the putative endophytic bacterium on the conservation of fraser photinia microshoots under *in vitro* conditions, contaminated microshoots were cultured on semi-solid sterile QL medium (medium pH was adjusted to 6.0 with 1 N HCl or 1 N NaOH before autoclaving), which contains 4.4 μ M 6-benzyladenine (BA) and 7 g/L agar and were stored at 6, 9, 12, 15, and 16 months under proliferation conditions (25 °C, 16/8 h photoperiod), without any subculturing or transferring a fresh medium. After conservation periods, the percentage (%) of green shoots, average length (mm) of the microshoots, dry and fresh weights (g) of microshoots were calculated in order to evaluate plant quality (Table 4.10, Figure 4.28). In addition, shoot apices from stored microshoots were “aseptically” taken and transferred on fresh QL medium which contains 4.4 μ M BA and it was evaluated that the proliferation of microshoots after storage.

In the presence of the putative endophytic bacterium, microshoots of fraser photinia were stored without subculturing at 25 °C for up to 9 months, with no reduction in the percentage of green microshoot formation (Table 4.10). However, a statistically significant reduction in the percentage of green microshoot formation was observed with the extension of the storage period. Microshoots stored for 15 and 16 months showed relatively low percentages of green microshoot formation (80.4% and 79.8%, respectively). But after storage at 25 °C for 16 months without transferring the plants to fresh medium and without slowing the plant metabolism by low temperatures, it is still possible to obtain 80% green shoots. In addition, there is no statistical difference between wet and dry weights of microshoots stored up to 16 months. Minimum wet and dry weights were obtained after storage for 6 months. This result shows that during storage the plant metabolism is active and increases its biomass at least up to 9 months.

After 6 months of storage under *in vitro* conditions, root formation observed in the microshoots. This root formation was also observed in microshoots stored for 16 months, although lower (14.7%). However, spontaneously rooted microshoots lose their vitality when they are taken to the acclimatization conditions.

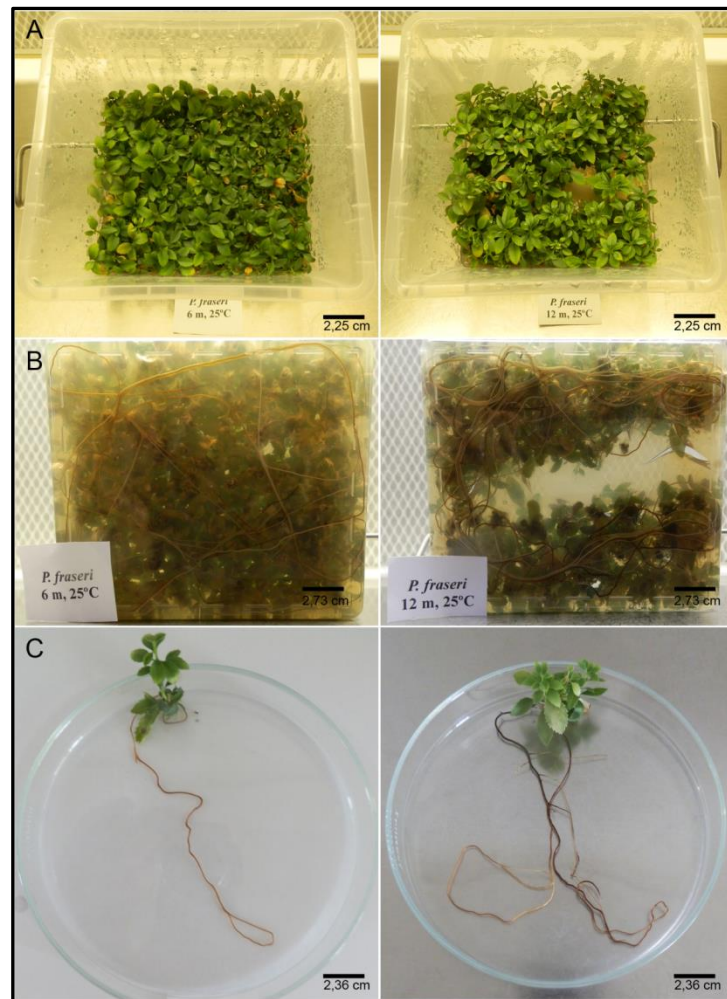


Figure 4.28: Post-storage status of the fraser photinia microshoots which are contaminated with the putative endophytic bacterium. A) The status of microshoots after storage for 6 months and 12 months, respectively, B) Rooting after storage for 6 months and 12 months, respectively, C) Rooted microshoots after storage for 6 months and 12 months, respectively.

Table 4.10: The effects of presence the putative endophytic bacterium on microshoot elongation and rooting during *in vitro* storage of microshoots^a.

Parameters	Storage period (months) at 25 °C				
	6	9	12	15	16
Green Microshoots (%) ^{bc}	100a	100a	95.5b	80.4c	79.8c
Microshoot Length (mm) ^{cd}	11.5±0.07b	12.9±0.05a	8.84±0.04c	11.5±0.19b	10.0±0.03c
Root Formation (%) ^{bc}	58.3a	35.5b	32.2b	36.7b	14.7d
Root/Microhoot ^{cd}	1.86±0.23b	3.50±0.87a	1.28±0.10c	3.00±1.08a	1.21±0.11c
Root Length (mm) ^{cd}	74.5±1.08b	54.8±0.78c	104.7±2.32a	85.9±1.22b	64.7±1.03d
Fresh Weight (gr) ^{cd}	3.69±0.09b	5.23±0.57a	5.34±0.28a	4.67±0.32a	5.93±0.94a
Dry Weight (gr) ^{cd}	0.79±0.09b	1.00±0.12a	1.00±0.10a	1.02±0.09a	1.04±0.02a

^a The data were collected 30 days after culture initiation. Each trial was made with at least 50 explants and the trials were repeated at least twice.

^b The same letters following the percentages show no statistical difference compared to the Post Hoc Multiple comparison test ($P \leq 0.05$).

^c The same letters following the averages show no statistical difference compared to the LSD test following ANOVA ($P \leq 0.05$; The average difference was analyzed horizontally).

^d Standard error of the mean $\pm$.

Although after 12 months storage, proliferation of the shoot apices from contaminated microshoots which stored at 25 °C *in vitro* conditions without subculturing began to decrease; proliferation was obtained in more than 93% of the shoot apices after 16 months storage (Table 4.11). Because maximum multiple microshoot formation was obtained with the longest storage period, more microshoot formation was achieved with the prolonged storage period. Thus, the highest SFC index (4.4) was obtained from shoot apices of microshoots which stored for 6 or 15 months. A low SFC index (3.5) was observed because of the statistically significant low percentage of proliferation with the extension of the storage period to 12 months. In addition, longer microshoots (4.7 and 4.4 mm, respectively) were formed after storage for 15 and 16 months.

Table 4.11: The effects of the putative endophytic bacterium on shoot apices proliferation of *in vitro* stored microshoots^a.

Parameters	Storage Periods (Months)				
	6	9	12	15	16
Proliferation (%) ^b	100.0a	98.9a	95.6b	98.8ab	93.3b
Microhoot/explant ^{cd}	4.40±0.17a	3.85±0.18b	3.51±0.14b	4.42.00±0.18a	4.44±0.13a
Microshoot Length (cm) ^c	3.46±0.14a	3.70±0.15a	3.56±0.01a	4.73±0.14a	4.41±0.14a
SFC Index	4.4	3.8	3.5	4.4	4.1

^a The data were collected 30 days after culture initiation. Each trial was made with at least 50 explants and the trials were repeated at least twice.

^b The same letters following the percentages show no statistical difference compared to the Post Hoc Multiple comparison test ($P \leq 0.05$).

^c The same letters following the averages show no statistical difference compared to the LSD test following ANOVA ($P \leq 0.05$; The average difference was analyzed horizontally).

^d Standard error of the mean $\pm$

^e SFC index of microshoots was calculated by multiplying proliferated explant to percentage of proliferated explant and dividing by 100.

There was not any significant effect of the putative endophytic bacterium on number of green leaves per misroshoot (Table 4.12). Besides, significantly higher number of abscised leaves and lower leaf senescence index were obtained with conserved microshoots up to 15 and 16 months.

Table 4.12: The effects of the putative endophytic bacterium on number of green/abscised leaves per microshoot and leaf senescence during conservation of photinia microshoots^a.

Parameters	Storage Periods (Months)				
	6	9	12	15	16
Green leaves/microshoot ^b	6.32a	5.81a	5.13a	5.15a	5.44a
Abscised leaves/ Microhoot ^b	0.58b	2.02ab	2.18a	2.33a	2.19a
Leaf Senescence Index ^c	2.46a	2.02ab	2.18a	1.74b	1.82b

^a Each treatment consisted of at least 50 explants and repeated at least twice.

^b Means followed by the same letter are not significantly different at $P \leq 0.05$ by the ANOVA, followed by the LSD test (i.e., mean significance is per horizontal lines).

^c Leaf senescence index of the microshoots were calculated according to $\sqrt{x/y+0.5}$ formula.

4.9. Effects of the Putative Endophytic Bacterium on Rooting and Acclimatization

Different results were obtained for root stimulation between control and contaminated microshoots cultured in medium containing IBA at different concentrations (Table 4.13). Maximum root formation was observed at the highest IBA concentration in non-contaminated microshoots. 66.6% of contaminated microshoots were rooted at 0.5 mg l^{-1} IBA concentration. The doubling of IBA concentration for contaminated microshoots caused decreasing of rooting to 43.3%. The minimum adventitious root number (1.2) was observed in contaminated microshoots at minimum IBA concentration (0.1 mg l^{-1}), even though tested IBA concentrations did not have a statistically significant effect on root formation per control microshoots. On the contrary, the longest roots were obtained from contaminated microshoots in this medium. Root formation was observed between 22-27 days in control microhoots, and between 27-32 days in contaminated microshoots. There was no difference in rooting time between contaminated and control microshoots except for the QL medium which contains IBA at minimum concentration.

Table 4.13: The effects of the putative endophytic bacterium on root formation in microshoots^a.

Parameters	IBA Concentrations (mg l ⁻¹)					
	0.1	0.5 Control	1.0	0.1	0.5 Contaminated	1.0
Root formation (%) ^b	40.0b	50.0b	70.0a	40.0b	65.0a	43.0b
Root/ Microhoot ^{cd}	2.0±0.6a	1.8±0.2a	1.8±0.3a	1.2±0.2b	1.6±0.1a	2.5±0.5a
Root Length (cm) ^{cd}	1.9±0.6b	1.7±0.4b	1.6±0.4b	3.2±0.6a	2.2±0.3b	2.3±0.3ab
Rooting time(day) ^e	22.3	26.2	27.6	32.0	27.6	26.5

^a The data were collected 30 days after culture initiation. Each trial was made with at least 50 explants and the trials were repeated at least twice.

^b The same letters following the percentages show no statistical difference compared to the Post Hoc Multiple comparison test ($P \leq 0.05$).

^c The same letters following the averages show no statistical difference compared to the LSD test following ANOVA ($P \leq 0.05$; The average difference was analyzed horizontally).

^d Standard error of the mean $\pm$.

^e Rooting time of microshoots was calculated according to $\Sigma (N_x T_x) / \text{rooting microshoot number}$. N_x is rooted shoot number; T_x is the number of days between the beginning of the test and the ending of the test.

5. DISCUSSION

All plants are associated with different microbial communities such as bacteria, fungi, and archaea. Endophytic microorganisms may be defined as organisms that colonize on plants without causing any harmful effects. Endophytes may promote plant growth, and exert other beneficial effects on them [Hardoim et al., 2015; Chaturvedi et al., 2016]. The most studied of these associations are the fungi-plant interactions. Although there are many studies on the isolation of endophytic bacteria from various plants, [Elvira-Recuenco and van Vuurde, 2000; Kuklinsky-Sobral et al., 2004; Belimov et al., 2005; Govindarajan et al., 2007; Mendes et al., 2007; Malfanova et al., 2011; Ren et al., 2011; Giongo et al., 2013; Ngamau et al., 2012; Mbai et al., 2013; Tariq et al., 2014; Kumar and Gera, 2014; Thanh and Diep, 2014; Szilagyi-Zecchin et al., 2014; Mareque et al., 2015; Afzal et al., 2015; Perez-Rosales et al., 2017; Singh et al., 2017], there are only a few studies on the detection of endophytic bacteria in *in vitro* plant tissue culture [Almeida et al., 2009; Dias et al., 2009; Abreu-Tarazi et al., 2010; Moraes et al., 2012; Faria et al., 2013; Quambusch et al., 2016; Pham et al., 2017].

In the GTU Plant Biotechnology Laboratory, as seen during the propagation of the fraser photinia microshoots, the presence of beneficial bacterium in plant tissue culture extend the period of subculture and in this way the plants can protect their vitality for a long time without adding any external additives to the medium. The microbiological, biochemical and molecular characterization of this putative endophytic bacterium, which was isolated from fraser photinia *in vitro* tissue culture, for the explanation its positive effect on fraser photinia is a first study in the literature.

Viable but non-culturable (VBNC) bacteria have very low metabolic activity. These bacteria do not divide despite being alive. But once they resuscitated they can be cultured [Colwell, 2000; Oliver, 2010]. The presence of the putative endophytic bacterium has been observed after about the fourth year of subculturing during the *in vitro* tissue culture studies of fraser photinia. It has been difficult to cultivate this bacterium as well as the fact that this bacterium was visible barely in the fourth year

of subculturing. It also takes a long period to reach the optimum growth of the putative endophytic bacterium compared to many other bacteria. These findings support the possibility that the bacteria will be a VBNC bacterium. Although the underlying reasons for the cultivation of VBNC bacteria is uncertain, it is believed that environmental changes such as pH, salinity, temperature, nutrients etc. alterations may have an effect on the cultivation [Thomas et al., 2008; Oliver, 2010]. There are a few studies that unculturable bacteria were isolated from various plants such as papaya, banana, and pineapple *in vitro* tissue culture [Thomas, 2007; Thomas et al., 2008; Abreu-Tarazi et al., 2010]. For instance, Abreu-Tarazi and co-workers use culture-independent molecular approach to reveal endophytic bacteria. Given the results of these studies, it is thought that there may be more than one endophytic bacterium in fraser photinia tissue culture.

Because the bacterium grew so slowly, the growth curve experiment could not be made. Another reason why the growth curve experiment cannot be done is the sensitivity of the putative endophytic bacterium to the oxygen. In this experiment, we tried to take culture sample from the bacterium daily and measure cell growth by UV spectrophotometer at 600 nm. Whenever we took bacterial culture sample, the bacterium exposed to oxygen. Thus, bacterial growth was affected poorly.

The carbon source must be added regularly for the viability of plants grown *in vitro* conditions [Pospisilova et al., 1987]. Although photosynthetic bacteria are typically aquatic microorganisms [Pfenning, 1967], firstly, it was thought that the beneficial effect of the putative endophytic bacterium on fraser photinia may be related to providing carbon source to plant by the bacterium. Therefore, the putative endophytic bacterium was investigated to determine whether it is a photosynthetic bacterium or not. As seen in Figure 4.2, the putative endophytic bacterium does not have any photosynthetic membrane according to TEM analysis. Therefore, the putative endophytic bacterium is not a photosynthetic bacterium and cannot provide carbon to plant by this way.

After understanding that the putative endophytic bacterium is not a photosynthetic bacterium, other mechanisms by which endophytic bacteria use to promote plant growth have been taken into consideration. As mentioned in the

literature review section, endophytic bacteria can affect plant growth positively by synthesizing various plant hormones, fixing nitrogen, producing siderophores etc.

Since iron is mostly in ferric ion form, soil microorganisms and plants cannot use it. Soil bacteria synthesize low molecular weight molecules called siderophores to use this iron. When siderophore molecule complexes with iron, this complex can be recognized and taken into cell by plants. Thus endophytic bacteria promote plant growth by synthesizing siderophores. In the thesis, the ability of the putative endophytic bacterium to synthesize siderophore was tested and the result was negative. On the other hand, bacteria cannot solubilize phosphorus. Phosphate is often insoluble in the soil and cannot be used by plants in this state. Endophytic microorganisms solubilize the phosphorus to make the plant use it. But the putative endophytic bacterium does not benefit the plant by this way.

Some bacteria may reduce NO_3 to NO_2 and even further to ammonia (NH_3) and nitrogen gas (N_2). This phenomenon, called denitrification, is accomplished by a variety of reductase (nitrate, nitrite, nitric oxide and nitrite oxide reductase) enzymes. These enzymes usually enable the use of nitrate as an electron acceptor in anaerobic conditions [Alexander and Strete, 2001]. In nature reduction of NO_3 occurs in the roots or leaves depending on the plant species and nitrogen conditions [Orsel et al., 2002]. Nitrogen is generally a limiting source of plant growth and development. Plants take nitrogen as ammonia or nitrate. Nitrate reduction by a bacterium is important in terms of nitrogen availability for the plant [Mbai et al., 2013]. According to the results obtained from the nitrate reduction test (Figure 4.15), the putative endophytic bacterium may reduce NO_3 to NO_2 and also to N_2 gas. In the tissue culture, prolongation of subculturing time and vegetative metabolic activities cause the pH reducing, and stimulate nitrate uptake instead of ammonium. Nitrate uptake causes the pH to rise again [George, 1993]. It has been determined that in various biochemical tests carried out within the scope of the thesis, the pH of the medium increase again because of basic byproducts that the putative endophytic bacterium produced. When these results are evaluated together, it is thought that the nitrate reducing capability of the putative endophytic bacterium may help the compounds containing nitrate in the medium to be metabolized by the plants, which was not

subcultured for longer periods. Thus, the plant may stay alive and grow without subculturing.

As mentioned in the literature review section nitrogen is one of the main nutrients that are necessary for all living organisms. However, it must be reduced to ammonia before it can be metabolized by plants. There are many studies about nitrogen fixing endophytic bacteria [James, 2000; Steenhoudt and Vanderleyden, 2000; Staal et al., 2001; Rajeswari and Mangai-Kasthuri, 2009]. Boddey et al., (1991) found that *Acetobacter diazotrophicus* is responsible for N₂ fixation with the plants. In another study it was found that wheat associated *Klebsiella pneumonia* has the N₂ fixation ability [Iniguez et al., 2004]. It was found that the putative endophytic bacterium has capability of fixing nitrogen. That means the putative endophytic bacterium may supply N₂ to plant.

It was found that the putative endophytic bacterium is catalase positive according to test results. Catalase degrades the H₂O₂, which is toxic to all living organisms, to O₂ and H₂O. The presence of catalase enzyme may protect not only the bacterium but also the plant against oxidative stress caused by tissue culture conditions.

Giberellins are tetracyclic diterpeneid acids which are involved in many developmental and physiological processes in plants such as seed germination, seedling emergence, stem and leaf growth. Among the 136 gibberellins, GA₃ that increase seed germination rate has a direct effect on stimulating the shoot extension and is also the most studied gibberellin [Bottini et al., 2004; Gupta and Chakrabarty, 2013]. In a study, which is conducted with PGPR, *Bacillus licheniformis* and *Bacillus pumilis* isolated from rhizosphere of *Alnus glutinosa* [L.] Gaertn. (Alder), it was measured that GA₃ concentration producing by these microorganisms. The results showed that these microorganisms produced 50-60 ng ml⁻¹ GA₃ [Gutierrez-Manero et al., 2001]. In another study which is conducted by Bastian et al., (1998), GA₃ production of *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* was analyzed, and was found that these microorganisms produced GA₃ at lower concentrations as about 12 ng ml⁻¹. *Bacillus siamensis* BE 76 is an endophytic bacterium which was isolated from Banana (*Musa* spp). It was investigated that the GA₃ production ability of this endophytic bacterium. This isolate produced high

amount of GA₃ (240 µg ml⁻¹) in medium supplemented with L tryptophan [Ambawade and Pathade, 2013]. The spectroscopic analysis of GA₃ showed that the putative endophytic bacterium produce this hormone at concentration of 28.92 µg ml⁻¹. *Bacillus subtilis*, known as a GA₃ producer, produced 29.36 µg ml⁻¹ of GA₃ in our experiment. The results show that the putative endophytic bacterium produces GA₃ at a higher concentration than most of the other studied endophytic bacteria.

IAA, one of the phytohormones, the most common form of auxin hormone, has an important effect on plant growth. IAA is also produced by some bacteria such as *Bacillus sp.*, *Aspergillus niger*, *Pseudomonas sp.*, *Acetobacter diazotrophicus* [Bilkay et al., 2008]. Bastian et al., (1998) investigated the IAA production capability of endophytic bacteria, *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae*. They found that both microorganisms produced IAA. *Herbaspirillum seropedicae* produces 7 ng ml⁻¹ IAA and *Acetobacter diazotrophicus* produces no more 32 ng ml⁻¹ of IAA. Xinxian and his colleagues (2011) isolated endophytic bacterial strains from *Sedum alfredii*. They observed that these strains produced IAA at different concentrations from 4.2 to 45.6 µg ml⁻¹. Besides, there are many studies in the literature about characterization of endophytic bacteria from several plants. In these studies, it was found many of the endophytic bacteria can produce IAA [Belimov et al., 2005; Sheng et al., 2008; Dias et al., 2009; Faria et al., 2013; Giongo et al., 2013; El-Awady et al., 2015]. According to test results, the putative endophytic bacterium that was isolated from fraser photinia microshoots produce 10 µg ml⁻¹ IAA.

The presence of auxin in the putative endophytic bacterium was also analyzed by PCR. It was seen ~950 bp single band on agarose gel electrophoresis. The PCR results show that *aux1* gene is present in the putative endophytic bacterium, albeit in different sizes than control microorganisms.

Cytokinin is another phytohormone that play role on modulation of cell division, seed germination, apical dominance, root elongation, differentiation of xylem and chloroplast, etc. Briefly, it promotes plant growth in many different ways. There are many studies that endophytic bacteria synthesize cytokinins [Farcia de Salamone et al., 2001] or cytokinin precursor [Pirttila et al., 2004] like plants. Endophytic bacteria that synthesize cytokinins are *Pseudomonas*, *Enterobacter*,

Staphylococcus, Azotobacter, and Azospirillum, etc. According to PCR results, the putative endophytic bacterium has *ipt* gene.

It has been reported that the promotion of plant growth and development may also achieved by endophytic bacterial genus, *Pseudomonas*, *Enterobacter*, *Staphylococcus*, *Azotobacter*, and *Azospirillum* [Arshad and Frankenberger, 1991; Leifert et al., 1994; Bashan and Holguin, 1997], which synthesize PGRs such as auxin, cytokinin, etc. The positive effect of the putative endophytic bacterium on fraser photinia growth and development may be a result of synthesizing PGRs by this bacterium. In microshoots, spontaneous rooting without additive auxin indicates the presence of endogenous auxin required for rooting [Saini et al., 2013]. This endogenous auxin may also be produced by bacteria other than auxin produced by the plant. It has been reported in the literature that growths of plants and explants in the sucrose-free medium of *in vitro* cultures are due to promoting the chloroplast growth and activity by methylobacteria through cytokinin production [Muromtsev et al, 1987]. The positive effect of the putative endophytic bacterium isolated from fraser photinia *in vitro* cultures may be due to the presence of cytokinin (*ipt*) or auxin (*aux1*) genes in its plasmid.

It is known that many bacteria such as *Agrobacterium tumefaciens*, *Rhizobium* sp., *Sinorhizobium meliloti*, and *Mesorhizobium loti* transfer genes to plants [Broothaerts et al., 2005]. *virD1* gene is found in the *vir* region of the *Agrobacterium tumefaciens* Ti plasmid and makes specific cuts at the T-DNA border. The presence of *virD1* gene in the putative endophytic bacterium shows that this bacterium may transfer genes to fraser photinia.

There are many studies on nitrogen fixing endophytic bacteria. In a study, it was investigated the effect of nitrogen fixing *Pseudomonas stutzeri* strain A15 on rice seedlings [Pham et al., 2017]. It was found that *P. stutzeri* A15 induced significant growth promotin compared to uninoculated rice seedlings. The putative endophytic bacterium may be good alternative with its ability to fix nitrogen as other nitrogen fixing bacterial strains.

PCR products of endophytic bacterium for *aux*, *ipt*, *nifH*, and *virD1* genes have been sequenced. However, the sequencing results were not enough for a good blast

analysis. Therefore, we could not find any similarity with other *aux*, *ipt*, *virD1* genes for any known sequences.

Genotypic identification of the putative endophytic bacterium by 16S rRNA sequencing did not show a high similarity to any known bacterial species [Şeker et al., 2017]. According to FAME analysis of the putative endophytic bacterium, the isolated strain has the highest similarity with *Ochrobactrum anthropi* and *Phyllobacterium myrsinacearum* (0.642 and 0.417, respectively). According to manufacturer's recommendation (Sherlock Microbial Identification System, version 6.0, MIDI, Inc, Newark, DE, USA), it is considered a good match if strain has a similarity index (SI) of greater than 0.500. These results are not high enough to classify the putative endophytic bacterium under *Ochrobactrum* or *Phyllobacterium* genus. Because, even though the results of some biochemical tests were common (i.e., Gram staining and catalase reaction) between endophytic bacterium and *Ochrobactrum anthropi* and *Phyllobacterium myrsinacearum*, their various biochemical characteristics (i.e., motility, oxidase, different sugar utilization as carbon sources) were strikingly different from our bacterium.

The culture medium used for the proliferation of plants *in vitro* conditions includes a carbon source, vitamins, and generally growth regulators at low concentrations. However, plants cultured *in vitro* conditions consume this medium in 30-40 days as they photosynthesize slightly. If the plants are not transferred to the fresh medium after the depletion of the nutrients, they will die from nutrient deficiency. The process of transferring the plant to fresh medium is called subculturing. For this reason, the plants should be subcultured for the renewal of the medium every 4-6 weeks depending on the plant species. Extension of the subculture period may be accomplished by the addition of some plant growth retardants such as abscisic acid [Kovalchuk et al., 2009], by reducing cultivation temperature from 25 °C to 15 °C [Negash et al., 2001] or 4 °C [Negri et al., 2000; Kovalchuk et al., 2009], or by adding some osmoticum such as mannitol [Negash et al., 2001; Divakaran et al., 2006]. However, the presence of putative endophytic bacterium provided the *in vitro* cultured Fraser photinia microshoots survive for a long time without reducing the temperature, adding any plant growth retardants, or subculturing. With this positive effect of the putative endophytic bacterium, microshoots of Fraser photinia could be

stored in standard culture medium and conditions for up to 14 months without losing their vitality. Beyond this, it has been observed that the shoot apices taken from microshoots that are stored for 16 months are also capable of providing a new and organizing growth following their transfer to a fresh medium.

The putative endophytic bacterium has a positive effect on growth of fraser photinia microshoots in many ways. The results showed that the putative endophytic bacterium might fix N_2 and reduce nitrate, produce IAA, GA_3 , and cytokinin, transfer genes to plant, balance the pH of the culture medium, and have catalase activity. These results may be the reason of this positive effect of the bacterium. Biofertilizers are environmentally friendly new fertilizer options that have no harmful effect on human, and animal health. Interest in biofertilizer is increasing recently. With these plant growths promoting properties the putative endophytic bacterium that isolated from fraser photinia is a promising candidate to be used as biofertilizer in agricultural applications.

6. CONCLUSIONS

All results obtained from the experiments that conducted in the scope of thesis are summarized as follows.

The putative endophytic bacterium, which was isolated from bacterial contamination in medium and microshoots of fraser photinia *in vitro* cultures, has been tried to be cultivated. It has been investigated that the growth of the bacterium in various media for this purpose. MPYE is the ideal growth medium for this bacterium and the growth of this bacterium become visible on fourth day of incubation in this medium.

Different temperatures (between 22-40 °C) and pH values were tested in the growth experiments of the putative endophytic bacterium. The bacterium was grown at temperatures at 22-34 °C and pH values at 5.8-10.0. Optimum growth temperature is 30 °C and optimum growth pH is 7.0 for this bacterium. It was also determined that the putative endophytic bacterium increase the medium pH and make the medium basic during growth on MPYE agar medium with BTB. This observation leads to the fact that the putative endophytic bacterium also makes basic the medium in which contaminated microshoots of fraser photinia were cultured.

According to other test results, the putative endophytic bacterium is gram negative, non-motile, non-spore forming. Microscopic images showed that the putative endophytic bacterial cells are irregular rod shaped 1-3µm x 0.5-0.8 µm in size and does not have the photosynthetic membrane structure.

According to biochemical analysis of the putative endophytic bacterium, it was found that the bacterium is FA, catalase (+), and has the capability of reduction NO₃ to NO₂. It was also determined that the bacterium cannot hydrolyze gelatin, lipid, protein, or starch. The putative endophytic bacterium use sucrose poorly, but do not use other sugars tested in 7-14 days of incubation.

According to antibiotic susceptibility test, it was found that the putative endophytic bacterium is resistant to Gentamycin (10 µg), Meropenem (10 µg), Cefepime (30 µg), Ciprofloxacin (5 µg), Ampicillin (10 µg), Aztreonam (30 µg), Ampicillin-sulbactam (20 µg), Levofloxacin (5 µg), Nitrofurantoin (300 µg),

Chloramphenicol (30 µg), Rifamycin (30 µg), Meticillin (5 µg), Vancomycin (30 µg), and Co-trimoxazole (25 µg) antibiotics. But then, It is sensitive to Erythromycin (15µg), Polymyxin B (300 U), Nalidixic acid (30 µg), Spectinomycin (100 µg), Streptomycin (10 µg), Co-trimoxazole (25 µg), and Tetracycline (30 µg)

As a result of determination the fatty acid methyl esters of the putative endophytic bacterium by GC, the highest fatty acids are found to be C18:1W7c (77.28%) and C16:0 (6.61%), respectively. It was also determined that C18:0 (4.61%), C17:0 iso (3.68%), C19: 0 CycloH8c (3.57%), C17:0 (2.62%) and C18:1w9c (1.63%) were also present in small amounts. When the results are evaluated according to the TSBA 60 database, it was found that the putative endophytic bacterium was similar to *Ochrobactrum anthropi* (Achromobacter Vd, CDC group Vd, 0,642) and *Phyllobacterium myrsinacearum* (0,417).

Genomic DNA of the putative endophytic bacterium was isolated for molecular characterization studies. In addition, according to the literature, it is also known that the vast majority of the endophytic bacterium has plasmids [Berde et al., 2010], plasmid DNA isolation of the putative endophytic bacterium was performed. As a result of these studies, a large plasmid was observed in the size of ~20 kb. The presence of auxin, cytokinin, nitrogen fixing, and virulence genes in the putative endophytic bacterium were investigated by PCR analysis using plasmid DNA, genomic DNA or bacterial culture as templates and universal primers specific to relevant genes. No band from PCR reactions in which bacterial genomic DNA used as template was obtained. Experiments using plasmid DNA or bacterial culture as template revealed that the endophytic bacterium carries sequences of *virD1*, *aux1*, *nifH*, and *ipt* genes.

It was observed that the *EcoRI*, *BamHI* and *HindIII* enzymes does not digest the plasmid DNA of the putative endophytic bacterium. When the plasmid DNA is digested with *PstI*, *MspI*, *MseI* and *HpaII*, the DNA is completely degraded.

The results of the experiment show that the putative endophytic bacterium has no adverse effect on the *in vitro* proliferation of the plant in the medium without cytokinin. In the medium containing cytokinin, it was determined that the contaminant increased the multiple shoot forming capacity of shoot apices. In

addition, no adverse effects of the endophytic bacterium have been observed on the rooting of these microshoots *in vitro* and on acclimatization *in vivo*.

When all results are evaluated, the presence of the putative endophytic bacterium in the culture medium and the synergistic effect with fraser photinia make it possible to store microshoots *in vitro* at 25 °C for long time periods. Thus, the subculturing period can be extended up to 16 months and the cost for subculturing is reduced. It is thought that this affect may be related the presence of cytokinin and auxin genes in the putative endophytic bacterium, and nitrate reduction, N₂ fixation capacity of the bacterium.

7. FUTURE PERSPECTIVES

The aim of this thesis is the biochemical and molecular characterization of the putative endophytic bacterium. The biochemical characterization of the bacterium was mostly revealed. Besides, the molecular characterization studies were conducted in line with the possibilities. The sequencing of whole bacterial genome could not be performed due to financial difficulties. The order sent to identify the plasmid DNA sequence could not be sequenced due to degradation caused by wrong transport conditions. Therefore, it is planned to carry out these sequencing and bioinformatics studies for better understanding of the association between bacterium and plant.

There are only a few studies on the detection of endophytic bacteria in *in vitro* plant tissue culture. The reason is that these bacteria cannot be cultured and thus they cannot become visible in the tissue culture media. For instance, Abreu-Tarazi and co-workers use culture-independent molecular approach to reveal endophytic bacteria [Abreu-Tarazi et al., 2010]. Given the results of these studies, it is thought that there may be more than one endophytic bacterium in fraser photinia tissue culture. It may be possible that just one of many could be cultured. Thus, the culture-independent molecular approaches may reveal new endophytic bacteria associated with fraser photinia.

Most endophytes isolated from plants were found to have antimicrobial activity and may protect their host plant against phytopathogens [Strobel, 2003; Yu et al., 2010]. It may be investigated the antimicrobial activity of the putative endophytic bacterium.

It is planned to investigate whether the putative endophytic bacterium is host specific. Biofertilizers are environmentally friendly new fertilizer options that have no harmful effect on human, and animal health. Interest in biofertilizer is increasing recently. With these plant growths promoting properties the putative endophytic bacterium that isolated from fraser photinia is a promising candidate to be used as biofertilizer in agricultural applications. If the bacterium is not host specific, then for using the bacterium as biofertilizer will be investigated.

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