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DETERMINATION OF NANOTIXICITY OF FUNCTIONALIZED TITANIUM DIOXIDE (TiO_2) NANOPARTICLES (NPs) TOWARDS *Rhizobium etli* BACTERIA IN SOIL

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
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November 2022

We certify that we have read this thesis and that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science

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Zaid Abbas Hadi ALHAJ OBAID

ABSTRACT

DETERMINATION OF NANOTIXICITY OF FUNCTIONALIZED TITANIUM DIOXIDE (TiO_2) NANOPARTICLES (NPs) TOWARDS *Rhizobium etli* BACTERIA IN SOIL

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Master of Science in Agriculture and Life Sciences

Advisor: Prof. Dr. Alpaslan KUŞVURAN

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November 2022

Emerging pollutants such as metal nanoparticles and TiO_2 nanoparticles have varying impacts on the survival of nitrogen-fixing bacteria in the soil. The health of the soil can be affected due to the harmful effects of these contaminants on plant-growth-promoting bacteria (PGPB), thereby impacting plant productivity. The produced TiO_2 NPs were examined using X-ray diffraction (XRD) and transmission electron microscopy (TEM) techniques. The toxicity of nano-titanium dioxide and the molecular effect of a gene on *Rhizobium* bacteria isolated from broad bean (*Vicia faba* L.) roots were studied. B cell receptor (BCR) was used to identify the isolates. To test the bacterial sensitivity to TiO_2, four holes were bored into the menthol medium using a 6 mm sterile drill. Titanium NPs were added at different concentrations (1225, 2500, and 5000 ppm) in the well; and after 24 hours, TiO_2 had no influence on bacterial growth but there was a high density of actively growing microorganisms. The relative gene expression of the *rhizobium* isolates (ropA) compared to the housekeeping gene (recA) was then measured. As a result, it was determined that the gene expression activity of the bacteria increased or decreased depending on the titanium concentration. It was concluded that titanium is a good option for increasing the activity and productivity of *rhizobium* bacteria, provided the appropriate concentration is determined.

2022, 50 pages

Keywords: Bacteria in soil, Functionalized, Nanotixicity, Nitrogen fixing, TiO_2

ÖZET

TOPRAKTA AZOT BAĞLAYAN *Rhizobium etli* BAKTERİSİNE KARŞI İŞLEVSELLEŞTİRİLMİŞ TiO_2 NPS (TİTANYUM DİOKSİT NANOPARTİKÜL)'NİN NANOTOKSİSİTESİNİN BELİRLENMESİ

Zaid Abbas Hadi ALHAJ OBAID
Tarım ve Yaşam BilimLeri, Yüksek Lisans
Tez Danışmanı: Prof. Dr. Alpaslan KUŞVURAN
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Metal nanopartiküller ve TiO_2 nanopartiküller gibi ortaya çıkan kirleticilerin bitki büyümesini teşvik eden bakteriler (PGPB) üzerindeki toksik etkileri toprak sağlığına zararlı olabilmektedir. Bu çalışmada; TiO_2 NPs'lerini karakterize etmek için TEM (transmisyon-geçirimLi elektron mikroskobu) ve XRD (x-ışını difraksiyon-kırılım) yöntemLeri kullanılmıştır. 25 nm partikül boyutuna ve -37 mV yüzey yüküne sahip kristal titanyum nanopartiküller, nano-titanyum dioksitin toksisitesi ve bakla (*Vicia faba* L.) köklerinden izole edilen bir genin *Rhizobium* bakterileri üzerindeki moleküler etkisi incelenmiştir. İzolatları tanımLamak için BCR (B hücre reseptörü) kullanılmıştır. Bakterilerin TiO_2'ye duyarlılığını test etmek için, 6 mm'lik steril bir matkap kullanılarak mentol ortamına dört adet delik açılmıştır. Farklı titanyum oranları (1225, 2500 ve 5000 ppm), 24 saatlik sürede, bakteri üremesi üzerinde herhangi bir etkide bulunmamış, fakat yüksek mikroorganizma yoğunluğuna yol açmıştır. Daha sonra, housekeeping geni (recA) ile *rhizobium* izolatlarının (ropA) nispi gen ekspresyonunu karşılaştırmak için ölçüm yapılmıştır. Araştırma sonucunda; bakterilerin gen ekspresyon aktivitesinin titanyum konsantrasyonuna bağlı olarak artış gösterdiği veya azaldığı tespit edilmiştir.

2022, 50 sayfa

Anahtar Kelimeler: Toprakta bakteri, İşlevselleştirilmiş, Nanotoksisite, Azot bağlama, TiO_2.

PREFACE AND ACKNOWLEDGEMENTS

In the name of Allah, the most gracious and merciful, all praise is due to Allah, the sustainer of the entire world, the origin of science and wisdom. May Allah's mercy and peace be upon our prophet, Mohammad, his relatives, and his companions.

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Zaid Abbas Hadi Alhaj OBAID
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CONTENTS

LIST OF SYMBOLS

℃	Celsius
g	Gram
m	Meter
µg	Microgram
µg L^{-1}	Microgram per liter
µg mL^{-1}	Microgram per milliliter
µL	Microliter
µm	Micrometre
mg	Milligram
mg L^{-1}	Milligram per liter
mL	Milliliter
mm	Millimetre
mV	Milivolt
ng μL^{-1}	Nanogram/microliter
nm	Nanometer
Ω cm^{-1}	Ohm centimeter

LIST OF ABBREVIATIONS

NPs	Net promoter score
OECD	Organization for economic co-operation and development XRD X-ray diffraction
TMDA	Tanzania medicines and medical devices authority
TiO_2	Titanium dioxide
TEM	Transmission electron microscopy
UV	Ultraviolet

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1. INTRODUCTION

In coatings and pigments, titanium dioxide (TiO_2) has been extensively employed since the early 1900s in various consumer and industrial applications. The photocatalytic characteristics of titanium dioxide nanoparticles ($nTiO_2$) have recently been used by the nanotechnology sector in a wider range of commercial and biological applications (Cherchi and Gu 2010). The release of $nTiO_2$ into aquatic habitats is expected as a consequence of the rising usage of the material. Wastewater treatment operations may create 5-15 g Ti L^{-1} effluent, which is comparable to estimated ambient quantities of titania nanoparticles (0,7-16 g L^{-1}) in typical Swiss environmental circumstances (Cherchi and Gu 2010).

Many impacts of nanoparticles on plants have been discovered in contemporary plant science research, including genotoxicity, phytotoxicity, and changes in plant shape and physiology. Chlorophyll in plants is affected both favorably and unfavorably by nanoparticles. The toxicity of plants caused by nanoparticles of various sizes, and the small size of nanoparticles is always the same. Depending on the NP (nanoparticle) type, size, dose, administration method, target plant species, and experimental circumstances, plants exposed to NPs (nanoparticles) have demonstrated positive, neutral, or negative effects. It has been established that some nanoparticles (such as TiO_2 NPs, Ag NPs, & ZnO NPs) exhibit antibacterial and antifungal activities against certain bacteria and fungi. Fungal hyphae and bacterial cells may be deformed and damaged by nanoparticles. As a consequence, soil microbial diversity and function may be adversely affected by the presence of NPs. As a result, soil microbial diversity and function may be compromised if NPs are present. The soil biota, resource complexity, and availability, as well as biophysical variability, may all have an impact on the impact of NPs on both root-colonizing and free-living bacteria in the rhizosphere (the region of the soil where root-microbe symbioses take place) (Tian and Kariman 2019).

Nanotoxicology is a branch of toxicology that focuses on the processes of human interaction with nanoparticles and the resulting pathogenesis. There is a wide range of

NP traits and attributes to deal with in this particular subject. Nanotoxicity levels have been estimated using several *in-vivo, in-vitro*, and *in-situ* models. For example, nanotoxicity can't be accurately determined due to NPs' biodistribution and absorption; both of which are dependent on the physical properties of NPs, including their size, surface chemistry, and dosage. Nanotoxicity processes, the entrances of NPs into human beings, and their nano-features are the major cause of most of the negative consequences of NPs exposure (Vlastou *et al.* 2017).

To better understand how emerging pollutants like metal NPs affect the survivability of nitrogen-fixing bacteria, this study looked at how TiO_2 NPs affect the soil. The toxic effects of these contaminants on PGPB, which affect plant productivity, can be harmful to the health of the soil. One of the most commonly created nanomaterials globally is TiO_2, which may also be the most released nanoform in soil.

2. LITERATURE REVIEW

2.1 Nanomaterials

2.1.1 Nano etymology

The word "nano," which means "dwarf" in Greek, is increasingly being used in scientific literature. Some of the nano-words that have lately entered dictionaries include nanometer, nanotechnology, nanoscale, nanoscience, nanotube, nanowire, nanostructure, and nanorobot. The idea of nanotechnology has been around for quite some time, but Richard Feynman's December 1959 lecture at the annual conference of the American Physical Society is often credited with coining the phrase, "*What would happen if we could arrange the atoms one by one the way we want them*?" (Vlastou *et al.* 2017) (Nobel Prize in Physics 1965), which was influenced by the speech "*There's Plenty of Room at the Bottom*" by the American Physical Society in the 1960s. He explained the chemist's method of placing atoms atom-by-atom, which results in a new material at either the atomic or macro scale. When Norio Taniguchi, of the Tokyo Science University, defined nanotechnology in 1970, he defined it as "*the technology linked with the diverse design and manufacture of structures by manipulating the size and form at the nanoscale scale*". When it comes to nanotechnology, 1-100 nm is the smallest size at which objects and phenomena are studied.

Many properties of matter are different at smaller scales than they are at larger scales, and this is where nanoscience comes into play, which focuses on the observation and study of nanometer-scale phenomena and how nanotechnology can be used to manipulate and control nanometer-scale matter (Yousaf and Ali 2008). The study of phenomena at the nanoscale scale is known as nanoscience. Nanometers are the standard unit of measurement for the size of atoms and molecules. To put it another way: the tiniest man-made gadgets meet the atoms and molecules of nature at the nanometer scale. When we say "nano," we usually imply the range from 10 to 9. Because one nanometer is one billionth of an inch, it is often used to describe the size of a single molecular particle. The human eye can't perceive anything smaller than a

nanometer. To view a 10 nm stone in your palm, you'd need an eye the size of a human hair, which isn't feasible. Whatever the case may be, a broad definition of "Nanoscience" would include everything with a minimum size of 100 nanometers or less (Yousaf and Ali 2008).

Precisely every U.S. federal agency, since 2000, has participated in the National Nanotechnology Initiative, with Departments and Agencies now working on nanotechnology projects. The National Scientific Foundation (NSF), an entity entirely accountable to the President of the United States and mandated to support the top basic science and technology initiatives, later sponsored around 20 Research Centers. The NSF was the primary American body responsible for implementing the NNI. Many media outlets (TV networks, the internet, etc.) and the public at large quickly became aware of "nanotechnology" as a term (Horikoshi and Serpone 2013).

2.1.2 Nanoparticles (NPs)

These are particle dispersions with a diameter of between 10 and 1000 nanometers. Crystalline or amorphous, NPs may transport liquids or gases on their surfaces. Since nano-particulate matter is distinct from the other four states of matter (solid, liquid, gaseous, and plasma), it should be considered as a distinct category of matter (large surface area and quantum size effects).

2.1.3 Classification of nanomaterials

Each dimension of a nanomaterial is categorized according to its length scale. For a nanomaterial to be considered nano, at least one dimension must be between 1 and 100 nanometers. All three dimensions (nano-particles, nano-films, and sheet-like nanomaterial) and one dimension (nanowires or fibers) may be found in nanoscale materials, as well as all three dimensions in macroscale materials (combinations of other nanomaterials, like a bundle of nanofibers, a bundle of nanosheets, etc). The graphic in

Figure 2.1 shows an identification approach for nanomaterials that is rather common (Tyohemba 2016).

0D: The nanoscale encompasses all three dimensions (nanoparticles).

One dimension at the nanoscale and two in at the macroscale make up one dimension (nanofibers, nanowires).

Nanoscale and macroscale dimensions are both 2D (nanosheets, thin films).

At the macroscale, everything is three-dimensional (nanostructures with nanomaterials).

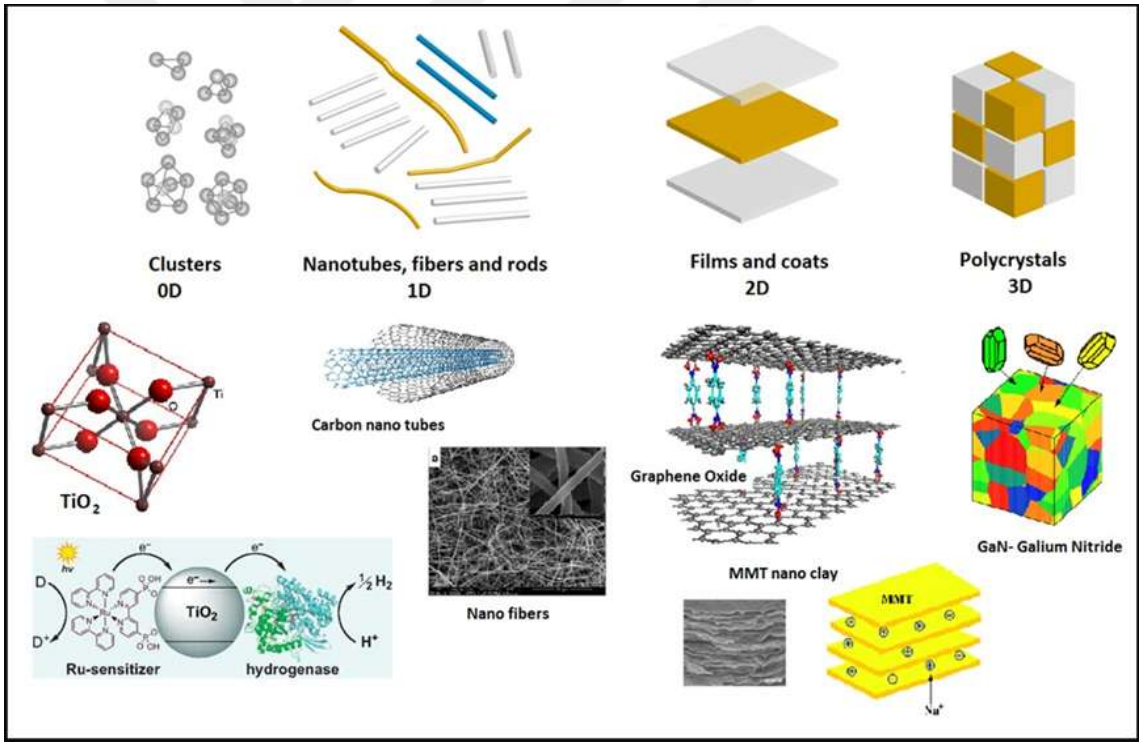

Figure 2.1 0-D, 1-D, 2-D and 3-D nanomaterials (Tyohemba 2016)

2.1.4 Importance of nanomaterials

Recently, these materials have gained interest due to their numerous properties, such as their unusual electrical, mechanical, magnetic, and optical properties, among others. The following are some examples (Pereira de Lyra *et al.* 2019).

The interest in nanophase ceramics is primarily driven by their higher flexibility at higher temperatures (Pereira de Lyra *et al.* 2019).

Nanostructured semiconductors have a long history of non-linear optical properties. The potential for quantum confinement events is a specific feature of silicon powders and silicon germanium quantum dots employed in infrared optoelectronic systems. Solar cells use window layers made of nanostructured semiconductors (Pereira de Lyra *et al.* 2019).

Nanosized metallic powders have made it feasible to create gas-tight materials, dense components, and porous coatings. In the electronics industry, the combination of ductility and cold-welding characteristics makes them excellent (Pereira de Lyra *et al.* 2019).

Magnetic nanophase materials have domain-like grains, whereas disordered walls at the borders represent the boundaries. Mono-domains are solitary, microscopic magnetic particles. In addition to their extraordinary superparamagnetism, ultra-small particles have unique atomic structures with discrete electronic states. Ferrofluids, high-density data storage, and magnetic refrigeration are just a few of the applications of magnetic nanocomposites (Pereira de Lyra *et al.* 2019).

Mono-or polymetallic nanostructured metal clusters and colloids are very useful in catalytic applications. Chemical transformations and electrocatalysis benefit greatly from heterogeneous catalyst (Cortex-catalytic) precursors, which have been discovered to be active, selective, and long-lasting (fuel cells). Catalysis may also be

enantioselective when chiral modifiers are applied to the surface of small metal particles (Pereira de Lyra *et al.* 2019).

Metal-oxide thin films are increasingly being used in the construction of gas sensors (NOx, CO, CO2, CH4, and aromatic hydrocarbons) with improved sensitivity and selectivity. Rechargeable batteries for automobiles and consumer products may be made using nanostructured metal oxide (MnO_2). Thin-film solar cells and dye-sensitized solar cells may both benefit from nanostructured titanium oxide porous films (Freitas *et al.* 2019). These polymer-based composites with high inorganic particle content may be used to produce photonic band gap structures (Pereira de Lyra *et al.* 2019).

2.1.5 B. Titanium dioxide NPs

Titanium dioxide NPs are non-toxic, and they are affordable, biocompatible, recyclable, and chemically stable. They have an effective antibacterial effect and can be used in several biological applications, such as medicines, cosmetics, whiteners, food coloring, and toothpaste. Chemical and biological approaches have been used in recent years to synthesize TiO_2 NPs.

While TiO_2 accounts for 70% of all pigment manufacturing globally, it also ranks among the top five non-porous polymers in consumer items. TiO_2 has long been used in prosthetic implants as an articulating component, notably in hip and knee replacements. Persistent immune responses to the implant material or implant deterioration are the most common causes of the failure of these implants. TiO_2 NPs are extensively manufactured and employed because of their excellent stability, anticorrosive characteristics, and photocatalytic capabilities. TiO_2 NPs' higher catalytic activity has been linked to both their large surface area and the fact that they are mostly anatase rather than rutile in composition. It is possible to use TiO_2 NPs in catalytic reactions such as semiconductor photocatalysis, water purification from hazardous industrial by-products, or as a photoactive material in nanocrystalline solar cells (Shi *et al.* 2013).

2.1.6 Physical properties of TiO_2 NPs

The unique properties of TiO_2 NPs made it suitable for many applications; it has boiling and melting points of 1843 ^oC and 2972 ^oC, respectively; it is insoluble in water even at the tiny particle size, and naturally develops as a solid. While other white materials may seem somewhat yellow under certain lighting conditions, TiO_2 does not because of the way it absorbs UV light and appears white but due to its high refractive index (the ability to scatter light). Titanium dioxide is even better than a diamond in dispersing light. As a result, titanium dioxide is a great material for use in aesthetic design. In addition, it has photocatalytic activity when exposed to UV radiation. Environmental purification, a variety of protective coatings, sterilization and anti-fogging surfaces, and even cancer treatment may all benefit from this technology (Shi *et al.* 2013).

2.1.6.1 Brilliant

It exhibits unmatched brilliance, color intensity, pearlescence, and transparency like no other material.

2.1.6.2 Resistant

Paint, films, and plastics are protected against deterioration by heat, light, and weathering using TiO_2.

2.1.6.3 Protective

TiO_2 is an essential sunscreen element because of its ability to scatter and absorb ultraviolet radiation, which helps keep skin safe from cancer-causing UV rays.

2.1.6.4 Powerful

TiO_2 is used in solar panels as a photocatalyst and to reduce air pollution.

2.1.7 Qualities of chemicals

Typically, titanium is mostly non-reactive and does not react with oxygen at room temperature. Additionally, it resists corrosion from chemicals like acids, chlorine, and others. However, titanium is very reactive at high temperatures. A material is said to be corrosive if it has a propensity to vehemently react or consume something. Titanium is highly flammable when heated in the presence of oxygen (Titanium 2019).

2.1.8 Nanotoxicity

The ability of a chemical to produce poisonous effects leading to serious biological harm or death after exposure to or contamination with that material is defined as "toxicity" as per the criteria of the OECD (Organization for Economic Co-operation and Development) criteria. Nanotoxicity is the word used to describe the toxicity that nanomaterials cause. More specifically, “nanotoxicity” is the branch of science that investigates the harmful effects of nanomaterials and nanostructures on biological systems. The main focus of nanotoxicological studies is the examination and evaluation of the toxic effects of nanomaterials in environmental or human health systems (Rauta *et al.* 2019).

2.2 Legumes

2.2.1 Leguminosae family

The legume family of flowering plants is one of the three biggest families of plants. About 750 genera and 19,000 species of herbs, shrubs, trees, and climbers make up the Leguminosae. The Mimosoideae, Caesalpinioideae, Swartzioideae, and Papilionoideae are four subfamilies within this huge family. About 80 species make up the Swartzioideae, a minor subfamily with little commercial value (Ahmed and Hasan 2014).

2.2.2 History of legume consumption

Legumes are plants that are thought to be the first to be domesticated by humans. Ancient Egyptian agriculture included lentils and fava beans, both of which are referenced in the Bible. In Neolithic (7000-8000 B.C.) fire pits in Turkey, charred seeds of peas, lentils, and vetches have been discovered. Peas (*Pisum* spp.) and a dwarf field bean were grown by lake dwellers in Switzerland between 4000 and 5000 B.C. Between 2000 and 3000 B.C., farmers in China started producing soybeans. More than 3,000 years ago, people in North America and Asia developed beans, soybeans, and other basic crops. As far back as 37 B.C., the Romans were using legumes in their pastures as well as improving the soil via their utilization (Ahmed and Hasan 2014).

2.2.3 Faba bean

Around the globe, numerous cropping strategies are used to cultivate the dry grain (pulse) and green grain/pod (green-manure legume) known as the faba bean (*Vicia faba* L.). The following are some of the ways that faba beans contribute to agricultural system sustainability (Fouad *et al.* 2013):

a) The first is its capacity to add nitrogen (N) to the system by biologically fixing it.
b) The second is that it diversifies production systems, which reduces disease, insect, and weed buildup and may even promote biodiversity.
c) Its ability to limit the use of fossil fuels.
d) Its ability to provide food and feed that are high in protein.

According to the fava bean's geographical origins in North Africa and Southwest Asia, as well as its widespread cultivation around the world, it belongs to the Central Asian, Mediterranean, and South American diversity centers. A Near Eastern origin was proposed by Cubero (1974), who put up the idea that Europe might be reached by four routes: the coast of North Africa to Spain, the Nile to Ethiopia, and Mesopotamia to

India. Although secondary diversity hotspots in Afghanistan and Ethiopia have been proposed, however, as per Hajjar and Hodgkin (2007), Central Asia has been reported as the origin of the species (Singh *et al.* 2013).

2.2.4 Significance of biological N_2 fixation for soil fertility

Using Biological Nitrogen Fixation (BNF) as a source of nitrogen is very efficient. In terms of annual terrestrial N_2, Burns and Hardy estimated that the BNF receives 139 million to 175 million tonnes, with 25 to 30 percent (or 35 million to 44 million tons) coming from symbiotic associations growing on arable land and another 30 percent (or 60 million to 72 million tons) coming from permanent pasture. This data, despite its dubious accuracy, shows the importance of BNF in crop and pasture systems and the enormity of the task required if BNF is improved to replace a portion of the 80 to 90 million metric tons of fertilizer-N applied annually and expected to be applied annually by the end of this decade. If we want to rescue our world, we need to stop harming land usage and start reversing land degradation significantly (Zahran 1999).

Lightning may also be generated naturally. In the long run, the N supply becomes more balanced as a result of long-term fixers' contributions to long-term N accumulation. The bulk of biological N2 fixation in agroecosystems is accomplished by symbiotic microorganisms such as *Rhizobium*, which infect the roots of legumes. About half of the world's total N fixation (including terrestrial and marine) is fixed by legumes (80 Tg), whereas the other half is fixed by industrial N fixation (the production of N fertilizer) (Zahran 1999). The amount of symbiotic N fixation may range from 20 to 200 kg of nitrogen per year. Root nodules are formed by legume roots to fix nitrogen dioxide and offer nutrients to the crop while also enhancing the soil's N supply.

2.2.5 Legume nodules

For legumes to fix nitrogen, a nodule must first develop in the soil (Figure 1). Soil microbes known as rhizobia penetrate the root and grow inside its cortex cells. The

plant provides the bacteria with all the nutrition and energy they need. There are obvious nodules within a week of infection (Figure 2.2). Depending on the legume species and germination circumstances, tiny nodules may be noticed in the field within 2–3 weeks after sowing. Inside, nodules that have not yet begun to fix nitrogen often have a white or gray hue. The color of nodules changes as they develop in size, as depicted in Figure 2.2, suggesting that nitrogen fixation has begun (Tyohemba 2016). Leghemoglobin (similar to hemoglobin in blood) is a component that is responsible for the pink or red hue of the nodules and regulates oxygen transport to bacteria (Figure 2.3).

Figure 2.2 A leguminous plant root (Tyohemba 2016)

Figure 2.3 The reddish-pink tint of the nodules linked to the roots of a legume may be seen when the nodules are ripped open (Tyohemba 2016)

2.2.6 Nodule organogenesis

The roots of the host plant exude phenolic flavonoid (Engelhardt *et al.* 1994) chemicals into the rhizosphere to induce nodule development (Figure 2.4). Because different rhizobia species react to different flavonoids in their exudate, it helps to define the uniqueness of the symbiotic connection. While most rhizobia species have a narrow host range, others have been shown to interact with a wide variety of legumes (Ferguson *et al.* 2010). Recent findings on the molecular mechanisms of nodule organogenesis have implications for understanding nod-signal activity, including further information on Nod-signal receptors and possible conserved lipo-chitin developmental regulators in legumes and non-legumes (Cohn 1998).

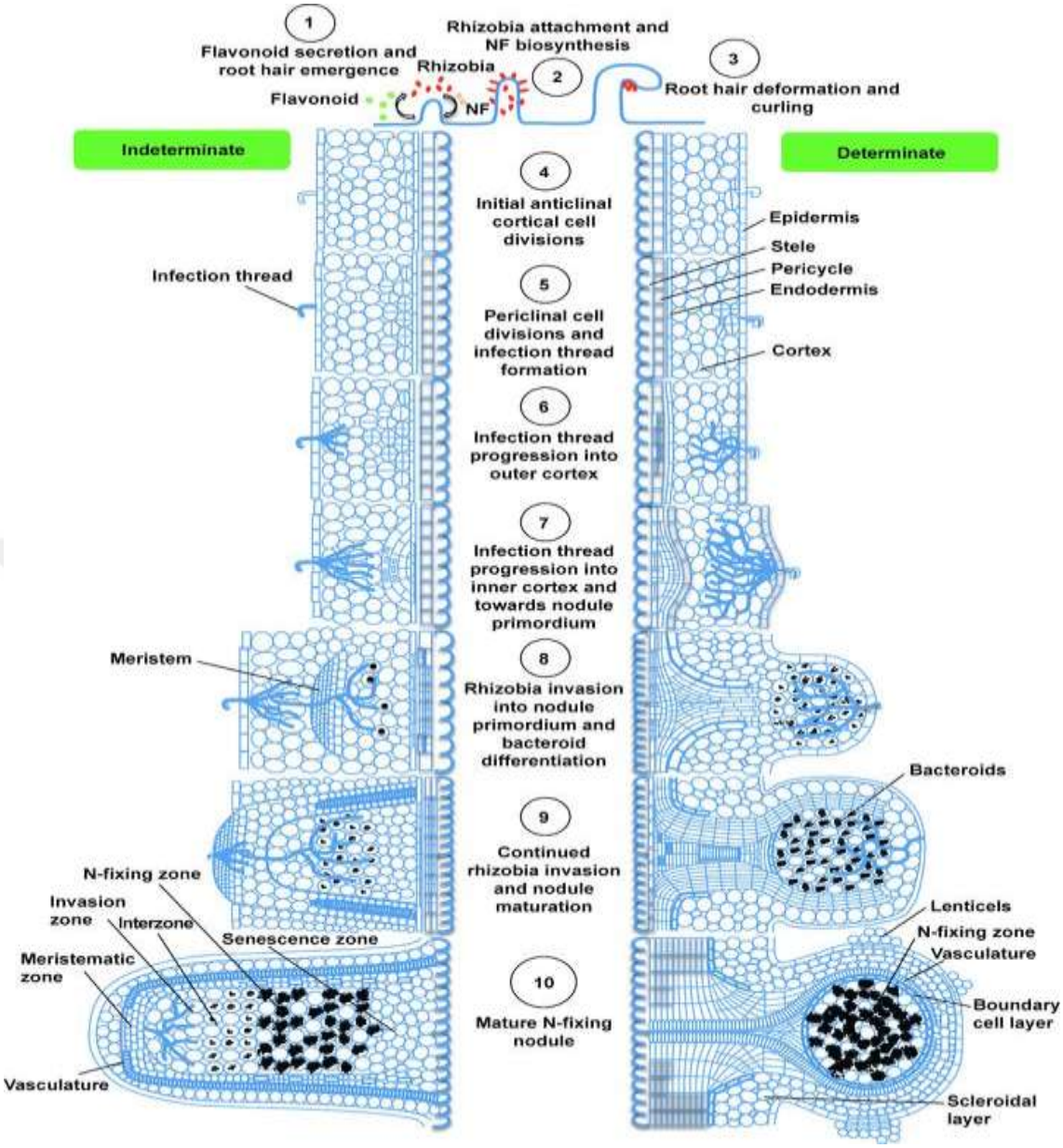

Figure 2.4 Development of legume nodules that are either indeterminate or determinate (Ferguson *et al.* 2010)

The *Rhizobium* and the plant have a unique and intricate relationship that results in nodulation. Rhicadhesin, a protein present on the surface of all leguminous plants, is often involved in the first attachment. Nodulation genes are triggered when these substances bind to the NodD protein. There are lipochito-oligosaccharides in *Rhizobium* that activate the early stages of nodulation in leguminous plants. Rhizobia's host specificity is governed by lipochito-oligosaccharide terminal sugar residues released by rhizobia (Parray *et al.* 2019).

Modifications in gene expression occur in both symbiotic partners within minutes to hours following the signaling conversation between bacteria and the host plant root. As the infection develops, both bacteria and plants experience significant morphological changes. When bacteria attach to the roots of a hair shaft, they disrupt the growth of the root hairs, leading to curls and branching. An infection thread is created by the plant at a high bacterial concentration on the hair surface, and this infection thread allows Rhizobia to multiply, divide, and infect plant tissue. During cell division, a nodule meristem develops in the root cortex. Infection threads, which have developed in newly split cells inside the nascent root nodule, release bacteria into infection droplets within the host cell cytoplasm. Bacteria are kept apart from the plant's cytoplasm by the plasma membrane, which encases the infection droplets. There are hundreds of distinct symbiosomes that are formed by interactions between the rhizobia and the surrounding symbiosome membrane. There is just one developed bacteroid in an indeterminate nodule like the one seen on peas or Medicago. The specialized symbiosome compartment created by this lengthy development process is where biological nitrogen-fixing takes place. Nodule development has become the subject of most high-quality studies (Haynes *et al.* 2004).

2.2.7 Factors affecting nodule formation in legumes

Many factors influence legume nodule formation (Soral and Bhayani 2021), such as:

a) External and internal factors, including ethylene-mediated autoregulation of root nodule formation, heat, soil acidity, drought, and availability of nitrate compounds.
b) The development of nodules and the symbiotic interaction between Rhizoids and plants are inhibited by nitrogen-rich soil. This is because plants have easy access to nitrogen.
c) The leaf tissues, which had particular chemicals that notify them anytime an infection thread opens, autoregulated the creation of nodules. As a result, they are controlled, among other thing, by sending signals to halt the opening of new infection threads,.

d) Internal effects of ethylene on the development of root nodules.

2.2.8 Types of legume nodules

Legume root nodules may be divided into two types: determinate and indeterminate. When it comes to the sort of root nodule that will develop, it all comes down to the type of host plant. Nodule growth, cell division, and ultimate form, all vary between the two types of nodules. Legume nodules such as pea, alfalfa, and vetch develop indeterminate nodules, while *L. japonicus* and soybean nodules are determinate. Interzone II-III is an area where microorganisms are consumed by plant cells as they move from Zone II to Zone III, and nitrogen fixation takes place in the indeterminate nodules (Zone IV). When it comes to the meristem, indeterminate nodules have an active one, while determinate nodules have an inactive one. Near the root attachment point, both Zone IV and the meristem may be found. Transcriptional and metabolic changes are zone-specific in the nodules formed between *Medicago truncatula* and *Ensifer meliloti* and *Ensifer medicae*, respectively. Zone V in alfalfa nodules has been the subject of significant debate. Proximal to the area of senescence, saprophytic intracellular bacteria are found here that do not become bacteroids (Cagliari *et al.* 2019).

2.3 Nitrogen Fixing Bacteria

For ecosystems to operate, soil organisms are responsible for a wide range of functions, including organic matter breakdown and nutrient cycling. Diazotrophs, a group of prokaryotes that includes the Leguminosae nodulating bacteria (LNB), play an important role in biological nitrogen fixation. An enzyme complex known as nitrogenase is used by prokaryotic organisms to convert atmospheric N into a form of nitrogen that diazotrophic organisms and plants can use (ammonia) (de Bruijn 2016). In the presence of ATP and reductant (electrons), it can fix ambient N into ammonia according to the following equation (Equation 2.1):

$$N2 + 8H + + 8e - + 16ATP \rightarrow 2NH3 + H2 + 16ADP + 16Pi \quad (2.1)$$

All species, including humans, may benefit from nitrogen fixation, which is the process of turning N from the atmosphere into ammonia and then N-containing organic compounds. For non-biological nitrogen fixation processes, such as lightning or the Haber-Bosch process, see below. Natural N fixation is the most common technique of N fixation. Biological N fixation fixes about 193×10^6 tons of N annually (Table 2.1) (Miyamoto *et al.* 2008). Almost all the fixed nitrogen is absorbed by plants; however, nearby non-legume plants may benefit from nitrogen "leakage" or "transfer" (0,03 – 0,06 ton hectare^{-1}). Plants that are nearby benefit from the nitrogen that is released by a legume's decomposition of its foliage (roots, leaves, and fruits).

Table 2.1 Biological and non-biological sources of yearly nitrogen fixation estimates. (Miyamoto *et al.* 2008).

Source of N fixation	**Nitrogen fixed (10^6 tons per year)**
Land	153
Legume	39
Non- legume	10
Others	104
Sea	40
Total biological	193
Lightning	9
Industry	85
Total non- biological	94

According to Sanyal *et al.* (2018), little nitrogen is returned to the soil following the harvesting of the grain of leguminous crops.

2.3.1 Types of nitrogen-fixing bacteria

Nitrogen-fixing bacteria are divided into two different groups - Anabaena and Nostoc among the many non-symbiotic (or free-living) bacteria, as well as genera such as *Azotobacter*, *Beijerinckia*, and *Clostridium* (blue-green algae). Some dicotyledonous plants (such as actinorhizal plants) and certain *Azospirillum* species (related to cereal grasses) include mutualistic bacteria such as *Rhizobium*, while *Rhizobium* and Frankia are examples of mutualistic fungi (Britannica 2020).

Agronomically significant systems' ability to fix nitrogen and how it might be improved. The primary agricultural and forestry N2-fixing methods. Agriculture and forests are home to all the main symbiotic N-fixing systems. They may be broken down into the following categories (Mokwunye and Vlek 2012): Rhizobium symbioses in legume plants that act as a food source for actinobacteria; Symbioses between Frankia plants and Gymnosperms (especially cycads); Free-living bacteria may also repair N in certain habitats; a kind of blue-green algae capable of fixing N (especially abundant in wetlands); Azospirillum, a heterotropic N-fixing bacterium found in the rhizosphere or plant residues (such as straw).

2.3.2 Characteristics of the rhizobia

Root nodule bacteria rhizobia (the fast-growing *Rhizobium* species and the slow-growing *Bradyrhizobium* species) are rod-shaped cells that vary in width from 0,5-0,9 microns to 1,2-3,0 microns long. They are gram-negative, have no endospores, and are motile. They have a single polar flagellum or two to six peritrichous flagella, but no endospores. Rhizobia, an aerobic chemoorganotroph, are easy to grow as long as they have access to oxygen. The ideal temperature and pH range for most strains are between 25-30 °C and 6,0-7,0. *Lotononis bainesii* nodulators generate red carotenoid pigments when grown on yeast-mannitol (YM) medium, unlike other rhizobia, which create white colonies. When grown in the conventional YM medium with bromthymol blue as the pH indicator, rhizobia also have certain unique growth responses. In the YM medium with bromthymol blue (pH 6,8), fast-growing rhizobia create an acid reaction, whereas slow-growing rhizobia cause an alkaline response (Somasegaran and Hoben 1994).

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Primers

The primers for PCR (Polymerase chain reaction) and Real-Time PCR were constructed in this study using information from the NCBI-Genbank database and primer3 plus (https://www.ncbi.nlm.nih.gov/genbank, https://www.primer3plus.com). Table 3.1 shows that Macrogen, a Korean company, provided these primers.

Table 3.1 PCR detection gene primers with their nucleotide sequence and product size

Gene	Seuences		Product size	NCBI reference
ropA pcr	F	TGGAAAACGACGACGCAAAC	507 bp	CP020906.1
	R	GCGCCATCACTTCGAATTCC		
ropA qpcr	F	AACGACTCTGATTGGGATGC	152 bp	M69214.1
	R	AGGTAAGCGGAGTCGAGGAT		
recA House Keeping gene	F	TATCTGCGCATTCGTTGACG	93 bp	KU904590.1
	R	GGCTGCGAGATCAAAAGGTTC		

1- The primers are specific for the amplification of the *Rhizobium etli* bacterium, not for their host (common beans).

2-Primer specificity (NCBI reference CP020906.1) was checked by NCBI primer blast as shown in the figure below.

3.1.2 Molecular study kits

Table 3.2 Presents the components of the Real-time PCR detection kits used in this study.

Table 3.2 The manufacturers and countries of origin of the real-time PCR expression kits utilized in this investigation

No.	Kit	Company	Country
1	**easy-BLUE™ Total RNA Extraction Kit**	**iNtRON**	**Korea**
	Trizol reagent 100mL		
2	**DNase I enzyme kit**	**Promega**	**USA**
	DNase I enzyme		
	10x buffer		
	Free nuclease water		
	Stop reaction		
3	**AccuPower® RocketScript™ RT PreMix**	**Bioneer**	**Korea**
	RocketScript Reverse Transcriptase (200U)		
	5X reaction buffer		
	dNTP 250µM		
	DTT 0.25Mm		
	RNase Inhibitor (1U)		
4	**RealMOD™ Green SF 2X qPCR mix**	**iNtRON**	**Korea**
	RealMODTM Green SF 2X qPCR mix (1mL)		

3.2 Methods

3.2.1 Purified water

Throughout this project, Milli-Q water (Millipore, UK) was used as a purification medium. Reverse osmosis was used to purify it using the Milli-Q water system (Millipore, UK). For example, at 25 °C, its surface tension is 71,9 mN m^{-1} and its resistance is measured to be < 18,3 ohms cm^{-1}.

3.2.2 Synthesis of TiO_2 nanoparticles (TiO_2 NPs)

The sol-gel process was modified following the method reported by Al-Awady and Paunov (2015) to produce the TiO_2 NPs. The pH of Solution A was adjusted by adding 1 M HNO_3 dropwise into 250 mL of Milli-Q water to achieve a concentration of 2,0, Isopropanol was also added to titanium tetrasiopropoxide to make Solution B (TTIP). The latter was added to solution A dropwise with vigorous stirring, resulting in a white turbid dispersion owing to TTIP hydrolysis. To get titania, the resulting suspension of Ti (OH_4) was heated at 70 ^oC for 20 h to produce a yellow-white precipitate, which was then filtered, washed with ethanol, and dried under vacuum for 2 h at 100 ^oC. Further experiments made advantage of the precipitate that had developed.

3.2.3 Sample preparation

An autoclaved, highly purified water solution containing 0,5 g of TiO_2 NPs was created using a digital sonicator (Branson Ltd.) with 40 percent amplitude for 5 minutes at 1 sec on/1 sec off pulse duration to disperse the samples. The TiO_2 NPs were kept in a fume cabinet for all experiments to prevent inadvertent spilling. The TiO_2 NPs were characterized using UV-Visible spectrometry and Transmission Electron Microscopy; they were also evaluated for particle size distribution and zeta potentials. The cytotoxic effects of TiO_2 NPs were also evaluated on *Rhizobium etli* cells (Figure 3.1).

Figure 3.1 A sonicator is used to melt titanium with sound waves

3.2.4 Bacteria isolation

The soil samples with plant roots were collected from flowering bean crops at the University of Baghdad's College of Agriculture. The soil was sandy, and the field was deficient in nutrients. After taking the samples, they were stored in plastic bags and refrigerated at -4 °C.

- To ensure that no part of the root was lost in the soil, the entire plant was uprooted, with the soil surrounding the rootstock.
- Water was used several times to wash the surface of the nodules with suspended dust to remove dirt particles. The surface sterile nodules were placed in a test tube containing 5 mL of sterile distilled water after being dipped for 30 seconds in a 0,1 percent mercuric chloride solution and washed ten times with sterile distilled water to eliminate toxic $HgCl_2$.
- The nodules were smashed with a sterile applicator to create a milky suspension of bacteria.

- The Mantol yeast extract medium was used. KH_2PO_4; 0,5% K_2PO_4; 0,5% yeast extract; 0,5% NaCl; 0,2% $MgSO_4.7H_2O$; 10 g mannitol; and 16,0 g agar are among the media components (Fentahun *et al.* 2013). This is accomplished by combining the ingredients in 1 liter of distilled water, shaking well to dissolve the materials, and then placing it in the autoclave for 15 minutes at 121 °C. The medium was allowed to cool slightly before being poured into petri dishes and refrigerated until use.
- The previously prepared bacterial suspension was cultured on yeast extract manthol medium and incubated at 28 °C for 7 days (Hamza and Alebejo 2017).
- The needle and node inoculation test was one of the techniques used to determine the presence of bacteria. A sample of bacteria was taken from the culture medium (YEMA) and cultured on another Petri dish, then incubated for three days at a temperature of 27-30 °C until the presence of bacteria was determined. It was then refrigerated until needed.

3.2.5 Polymerase chain reaction (PCR)

The following procedures were used to achieve a confirmatory molecular detection-based amplification using the PCR method (Chen and Kuo 1993). Nitrogen-fixing bacteria (*Rhizobium etli* isolates) were used to get the bacterial genomic DNA using the Presto^{TM} Mini gDNA Bacteria Kit in accordance with the manufacturer's instructions (Figure 3.2).

3.2.10. 1 Sample preparation

a) 1 mL of the cultivated bacteria that had been incubated for 18 hours (up to 1×10^9) was transferred into a 1,5 mL microcentrifuge tube.
b) The tube was centrifuged for 1 minute at 10,000 rpm before discarding the supernatant.

3.2.10. 2 Cell lysis steps

a) After adding 180 µL of GT buffer to the tube and using a vortex to suspend the cell pellet, the next step is the addition of Proteinase K (20 µL), followed by incubation of the resulting solutions for 10 min at 60 ^{o}C.
b) Every three minutes, the mixture tubes were inverted during the incubation periods.
c) Each tube received 200 µL GB buffer, which was then mixed in a vortex for 10 seconds. The tubes were then incubated at 60 ^{o}C for 10 minutes, with 3 minutes between each inversion.

3.2.10. 3 DNA binding steps

a) 200 µL of pure ethanol was added to the mixture, followed by vigorous agitation; the resulting precipitates were broken up via pipetting.
b) All mixtures (including any precipitate) were transferred to a GD column, which was positioned in a 2 mL collection tube.
c) The mixture was centrifuged for one minute at 10,000 rpm. The flow-through-containing 2 mL collection tubes were discarded, and a fresh 2 mL collection tube was used to hold the GD column.

3.2.10. 4 Washing steps

a) The GD column received 400 µL of W1 buffer, which was then centrifuged for one minute at a speed of 10,000 rpm. The GD column was then put back into the 2 mL collection tube after the flow-through was discarded.
b) The GD column received the addition of b-600µL Wash Buffer. then spun the mixture for one minute at 10,000 rpm. The GD column was then put back into the 2 mL collection tube after the flow-through was discarded.
c) The column matrix was dried, and the tubes were further centrifuged at 12000 rpm for 2 min.

3.2.10. 5 Elution steps

a) A clean 1,5 mL microcentrifuge tube was used to transfer the dried GD column, and 100 µL of pre-heated elution buffer was added to the column matrix. The absorption of the elution buffer by the matrix was ensured; the tubes were allowed to stand for about 3 min.
b) The tubes were centrifuged for one minute at 10,000 rpm to elute the purified DNA.

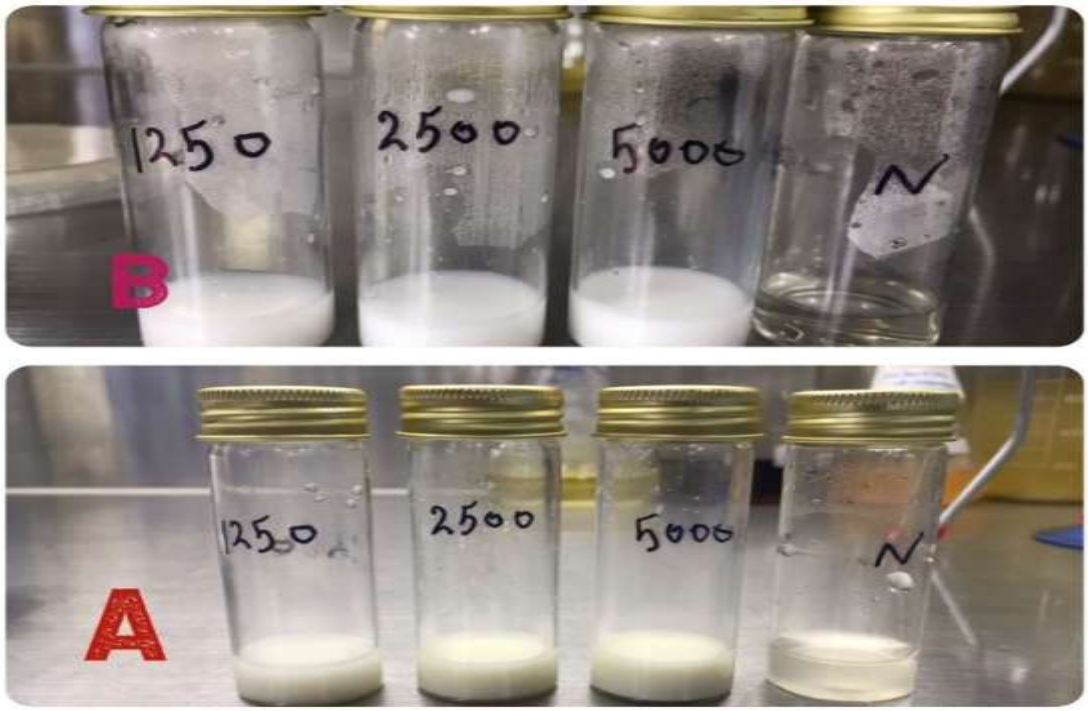

Figure 3.2. *Rhizobium etli* used in the (PCR) test: A) before growth, B) after growth

3.2.6 Estimation of extracted total DNA

The Nanodrop (Thermo Scientific NanoDrop Lite UV Visible Spectrophotometer, USA), which measures DNA concentration (ng L^{-1}) and checks RNA quality at 260/280 nm, was used to examine the extracted total DNA as follows:

1. The proper program was selected after launching the Nanodrop software (Nucleic acid, DNA).

2. The measurement pedestals were repeatedly cleaned with a dry wipe,and 2 µL of free nuclease water was carefully pipetted and placed on the lower measurement pedestals to blank the apparatus.

3. A 1 µL DNA sample was measured after the Nanodrop sampling arm was lowered. A nanodrop was used to quantify the DNA content (ng L^{-1}) and purity at 260/280.

3.2.7 PCR master mix preparation

The PCR master mix components in Maxime PCR PreMix tubes were supplemented with all other components required for DNA amplification by PCR, such as those specified above in the table (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9,0, KCl, $MgCl_2$, and loading dye). The PCR tubes were spun in an Exispin vortex for three minutes at a speed of 3000 rpm before being placed in a T100 PCR Thermocycler for analysis (BioRad-USA).

This master mix was created following the manufacturer's instructions, as shown in Table 3.3 for all genes using a Maxime PCR PreMix kit.

Table 3.3 The usual PCR master mix procedure

PCR Master mix	Volume
Ten nanograms of DNA template dilution	5µL
Forward primer (10pmol)	1µL
Reveres primer (10pmol)	1µL
PCR water	13µL
Total volume	20 µL

3.2.8 PCR thermocycler conditions

Temperatures were computed for each gene using the Optimase ProtocolWriterTM program and performed using a conventional PCR thermocycler as shown in Table 3.4.

Table 3.4 PCR thermocycler conditions protocol

Protocol type	Simple 3-step PCR protocol
Step 1	95 ºC, 2 min.
Step 2	95 ºC, 30 sec.
Step 3	58,3 ºC, 30 sec.
Step 4	72 ºC, 20,0 sec.
Step 5	Repeat steps 2-4 29 more times
Step 6	72 ºC, 5 min.
Step 7	4 ºC, forever

A gel electrophoresis technique was used to examine the PCR results, as follows:

1. 0,5X TBE was dissolved in the microwave for 5 minutes, and then the gel was allowed to cool at 50 °C for an additional 5 minutes.
2. Then, 3 mL of the staining agent, and ethidium bromide was added.
3. Carefully remove from the tray after the agarose gel solution had solidified for 15 minutes at room temperature (for the comb to be removed from the tray).
4. 0,5X TBE buffer was added to the gel tray, which was then placed in the electrophoresis chamber and clamped down.
5. Each well received 10 µL of the PCR product, with 5 µL (DNA marker Ladder) added to the first well. Then, 100 volts of current at 80 Hz was applied for an hour.
6. The PCR reaction results were visualized using a UV Transilluminator.

3.2.9 Well-diffusion agar

After 24 hours of incubation, the harmful effects of various concentrations of TiO_2 NPs suspension (1250, 2500, and 5000 g/mL) were investigated on *Rhizobium etli* using the well diffusion agar technique. This procedure was carried out using plates with 6 mm agar wells, which were subsequently filled with 10 µL of a specified concentration of TiO_2 NPs in aliquots. The zone of inhibition was measured after a 24-hour incubation period, as suggested by Begum *et al.* (2015). Four wells (6 mm) were bored in the media using a sterile cork borer. To provide an accurate measurement of the zones of inhibition, the plates were infected and then incubated for 24 hours at 27 ºC. For each concentration, three samples were made (Figure 3.3).

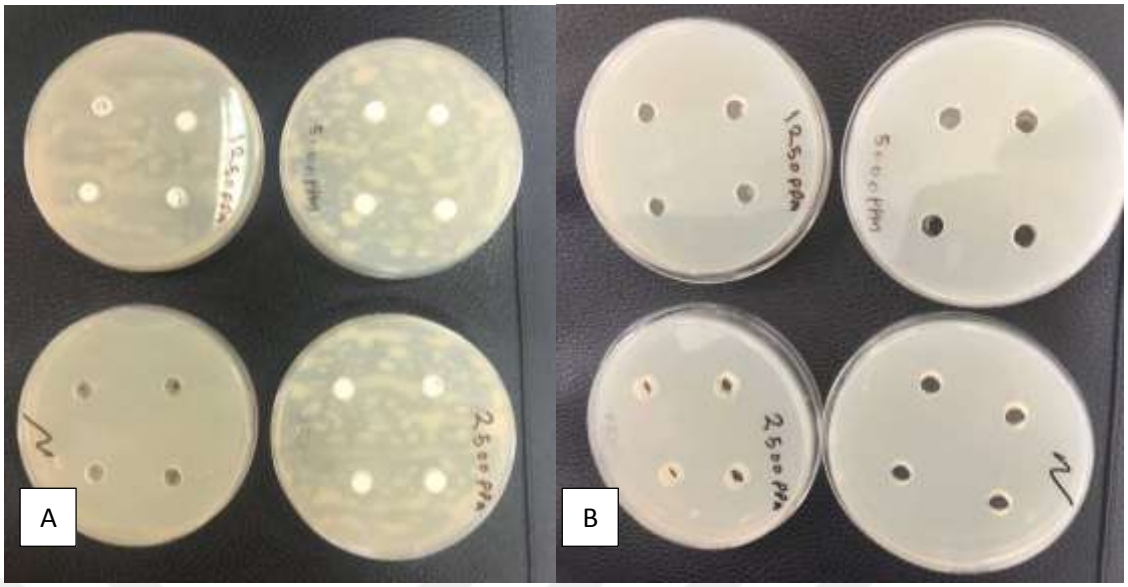

Figure 3.3 Using the well diffusion agar technique: A) before growth, B) after bacteria growth

3.2.10 Quantitative reverse transcription real-time PCR

The ropA gene by housekeeping (reC) in (*Rhizobium etli*) isolates were quantified, and the relative gene expression analysis was carried out using the quantitative Real-Time PCR technique. According to Farrell Jr. (2009), the procedure used involved the following steps:

3.2.10. 6 Total RNA extraction

The easy-BLUETM Total RNA Extraction Kit was utilized following the manufacturer's instructions, to extract the total RNA from nitrogen-fixing bacteria (*Rhizobium etli)*.

1. The first step is centrifugation at 13000 rpm for 1 minute for the collection of the bacterial cells (OD600:0,8-1,0) from the Luria Bertani broth that had been incubated at 37 ℃. The supernatant was then removed.
2. The bacterial pellets were violently vortex-suspended in 1 mL easy-BLUE (Trizol reagent) for 10 second.

3. Each tube was filled with 200 mL of chloroform and forcefully shaken for one minute.
4. The mixture was kept on ice for 5 minutes and after that, there was 15 minutes of centrifugation at 13000 rpm and 4 ℃.
5. Microcentrifuge tubes of 1.5 mL capacity were used to transfer the supernatant into a fresh isopropyl-containing tube, followed by incubation for 10 min at 4 ℃ with occasional flipping of the tube (four or five times). The samples were centrifuged at 13000 rpm and 4 ℃ for 10 minutes.
6. When the liquid was again vortexed, 1 mL of 80 percent ethanol was added and centrifuged at 13000 rpm at 4℃ for 5 min.
7. The RNA pellet was left outside to dry since there was no supernatant. Each sample was dissolved by adding 100 mL of free nuclease water. A sample of RNA was obtained and then kept at -80 ℃ until needed.

3.2.10.1 Estimation of extracted total RNA

A nanodrop was used to measure the concentration and purity of the total RNA extracted from the cells using the following approach:

1. Open the nanodrop app and choose the appropriate application (Nucleic acid, RNA).
2. Dry wipes were used several times to clean the measurement pedestals. In the next step, a blanking agent (2 mL of free nuclease water) was pipetted into the bottom measurement pedestals.
3. The measurement of 1 nmol of RNA was carried out using the Nanodrop sampling arm.

3.2.10.3 DNase I treatment

A DNase I enzyme kit (Promega, USA) was used to eliminate genomic DNA traces from the eluted total RNA. This was done by setting up the DNase digestion reaction as in Table 3.5.

Table 3.5 DNase I enzyme kit

Mix	Volume
Total RNA 1µg	10µL
DNase I enzyme	1µL
10X buffer	4µL
DEPC water	5µL
Total	10µL

1. Incubate at 37 °C for 30 minutes.
2. Add 1µl of RQ1 DNase Stop Solution to terminate the reaction.
3. Incubate at 65 °C for 10 minutes to inactivate the DNase.

3.2.10. 4 cDNA synthesis

cDNA was synthesized from mRNA transcripts using the AccuPower® RocketScriptTM RT PreMix following the manufacturer's instructions as shown in Table 3.6.

Table 3.6 cDNA synthesis

RT mix	Volume
Total RNA 100µg	10µL
Random Hexamer primer (50pmol)	1µL
DEPC water	9µL
Total	10ul

a) cDNA Protocol

1. Before using, thaw the primer, DEPC-water, and total RNA.
2. Fill the AccuPower® RocketScriptTM RT PreMix tubes with total RNA and the appropriate primer (oligo dT, random primer, or particular primer).
3. Fill the AccuPower® RocketScriptTM RT PreMix tubes with DEPC-water in amounts of 20 l (K-2101, K-2102) or 50 l. (K-2103, K-2104). The dried pellet should not be calculated.
4. Using Bioneer's ExiSpin Vortex/Centrifuge or by pipetting up and down repeatedly and momentarily spinning down, fully dissolve the vacuum-dried pellet.

5. Execute the reaction under the aforementioned conditions.
6. After amplification, keep the reaction at℃; the sample can be kept at -20 ^{o}C until needed.

3.2.10. 5 Real-Time PCR (qPCR) master mix preparation

RealMOD Green SF 2X qPCR mix Kit) based on SYBER green dye amplification in Real-Time PCR equipment was used to make the qPCR master mix:

For target and housekeeping genes in a quantitative polymerase chain reaction, the qPCR master mix for target genes and housekeeping genes is shown in Table 3.7.

Table 3.7 Standard master mix procedure for quantitative PCR

qPCR master mix	Volume
cDNA template (10 ng)	5µL
Forward primer(10 pmol)	1 µL
Reverse primer (10 pmol)	1 µL
qPCR Master Mix	10 µL
Nuclease free water	3 µL
Total	20 µL

All these were done before inserting the white plate strip tubes with the components of the master mix into a MiniOpticon Real-Time PCR system, which was combined using an Exispin vortex and centrifuged for five minutes.

Optimase ProtocolWriterTM online was used to calculate primer annealing in the qPCR thermocycler according to the kit's instructions and as shown in Table 3.8.

Table 3.8 qPCR thermocycler conditions

Protocol type	Simple 3-step PCR protocol
Step 1	95 ºC, 2 min.
Step 2	95 ºC, 30 sec.
Step 3	58,3 ºC, 30 sec.
Step 4	72 ºC, 20,0 sec.
Step 5	Repeat steps 2-4 29 more times
Step 6	72 ºC, 5 min.
Step 7	4 ºC, forever

These equations were derived from the qPCR data obtained by (the Livak technique) as stated by (Livak and Schmittgen 2001), using the following findings for both target and housekeeping genes:

CT = CT (Test) (target gene, test) CT – (HKG gene, test)

It is equal to CT (target gene, control) – CT (HKG gene, control)

CT = CT (Test) – CT (Results) (Control)

Target / HKG = 2-CT fold change. ΔΔCT.

4. RESULTS AND DISCUSSION

4.1 Synthesis and Characterization of TiO_2 NPs

The approach described in the literature was used to generate nano-sized titania nanoparticles in this work (Al-Awady *et al.* 2015). According to the Sol-Gel technique, titanium NPs were created by hydrolzingof titanium isopropoxide in an acidic medium, then condensing the generated solution for 18 hours at 70 ^{o}C to produce a blue-white suspension, which was washed with ethanol and dried at 100 ^{o}C for 24 h. Precipitation of yellow-white titania nanoparticles was observed.

The zeta potential and particle size distribution of the synthesized titania nanoparticles were measured using the dynamic light scattering method. The average particle diameter is shown in Figure 1, whereas the surface charge is shown in Figure 2. The tiny titania NPs (TiO_2) were demonstrated in Figure 4.1, while Figure 4.2 showed the nanosize of the TiO_2 NPs (25 nm) with an average potential of -37 mV. These findings show that the produced TiO_2 NPs have a long shelf life and are stable at the nanoscale.

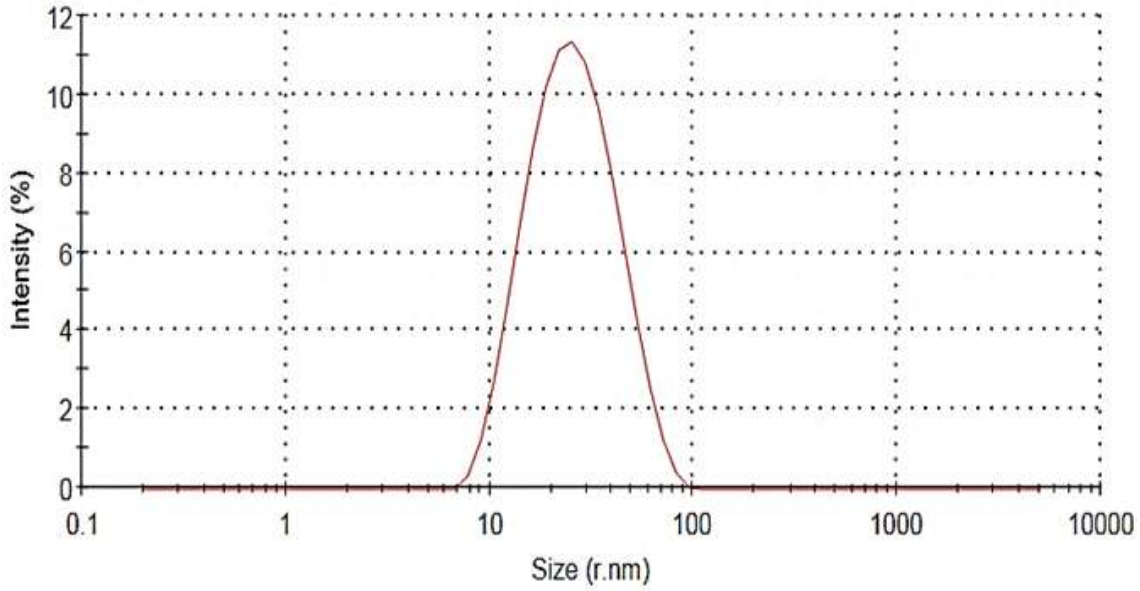

Figure 4.1 The particle size distribution of titanium dioxide nanoparticles synthesized by using titanium isopropoxide, isopropanol, and distilled water adjusted to pH 2 and dispersed with water at 30% amplitude for 5 minutes.

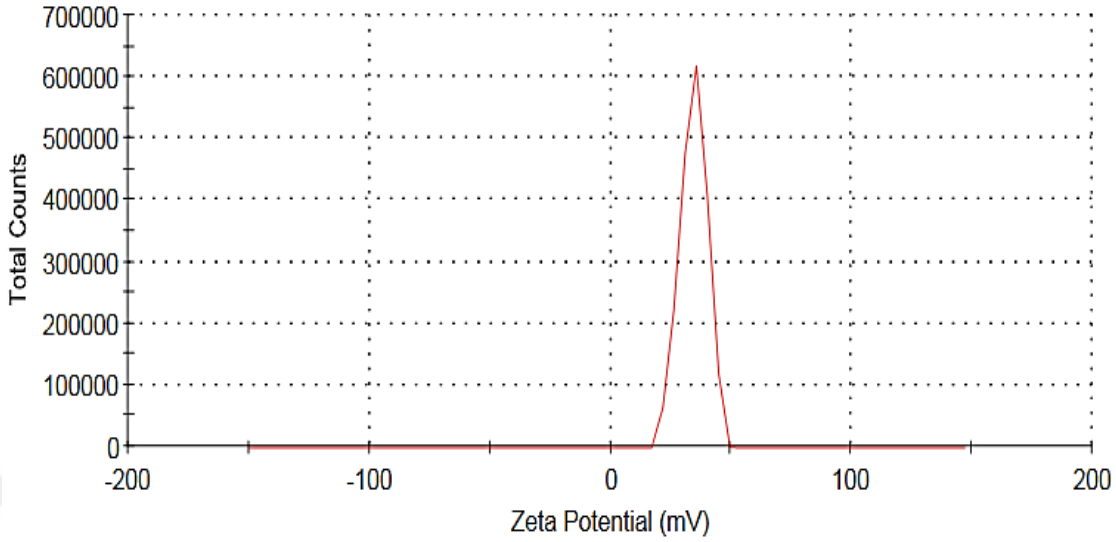

Figure 4.2 Zeta potential measurement of titanium dioxide nanoparticles synthesized by using titanium isopropoxide, isopropanol, and distilled water adjusted to pH 2 and dispersed with water at 30% amplitude for 5 minutes

The produced crystalline titania nanoparticles' crystallite size and shape were studied and measured using X-Ray Powder Diffraction. The Scherrer function is used to compute the crystallite sizes of titanium nanoparticles in Figure 4.3. Titanium nanoparticles with a crystallite size of 5 nm are illustrated in the image. A transmission electron microscope (TEM) was used to examine TiO_2 NPs, as shown in Figure 4.4 which shows that the nanoparticles generated from titania are virtually spherical in terms of morphology and crystalline in form, which is consistent with the XRD data.

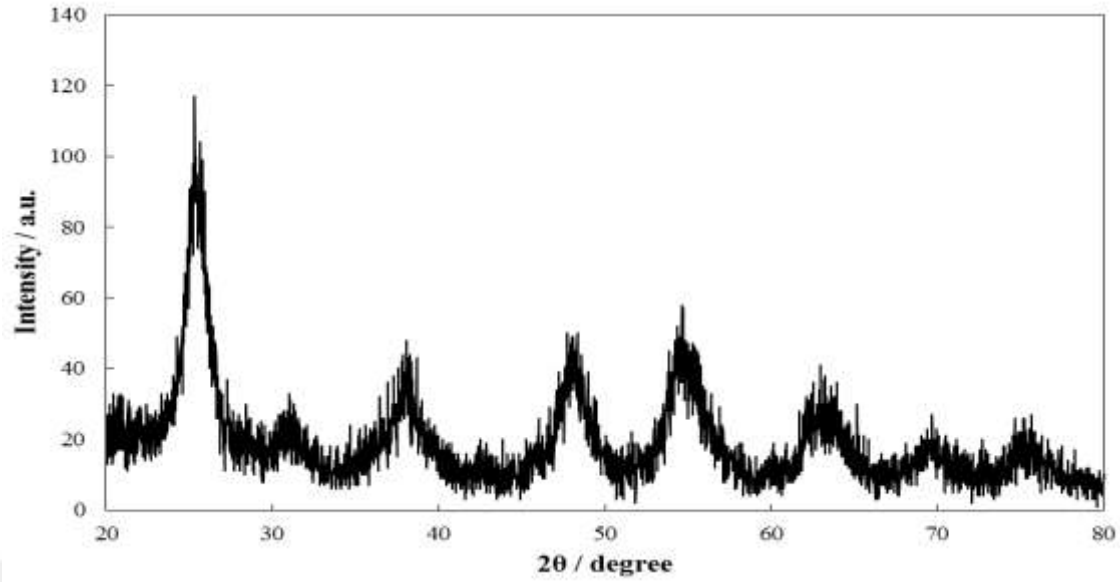

Figure 4.3 X-ray diffraction patterns of the synthesized titania nanoparticles synthesized using the sol-gel method

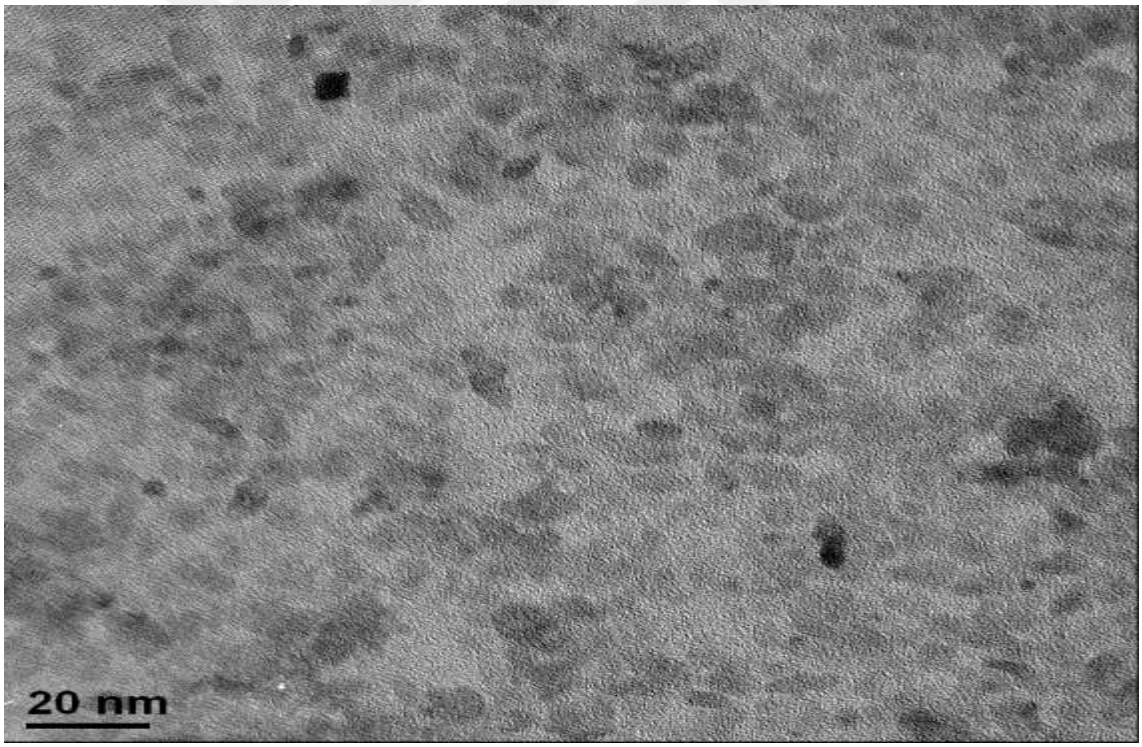

Figure 4.4 Transmission electron microscope of titania nanoparticles prepared by using the sol-gel method

4.2 Sample Analysis

Beans, legumes, and leguminous plants of the legume family, or legumes, were cleaned by washing with running water several times and then rinsed with a mild CI or commercial express solution comprising 6% CI and 20% dilution. To remove any remaining sterile solution, it was rinsed three times with sterile distilled water. To obtain the necessary bacterial suspension, 400 antifungal mg and 10 mL of sterile distilled water were added to the powder, which was then placed in a sterile baker, followed by the addition of 10 mL of sterile distilled water to the powder. The sample was kept in the refrigerator as a vaccine source for subsequent experiments, as shown in Figure 4.5.

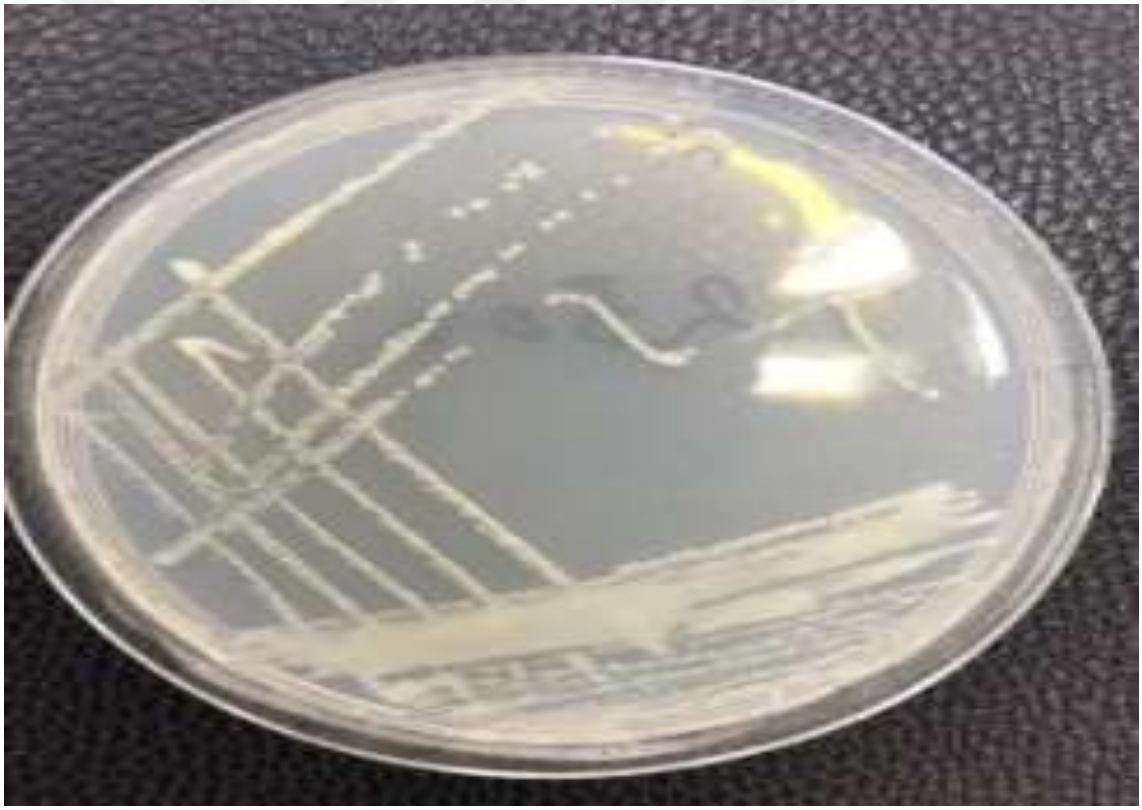

Figure 4.5 Bean root knot bacteria sample

4.3 PCR Diagnostics

Rhizobium etli PCR bacteria were molecularly diagnosed, as illustrated in Figure 4.6.

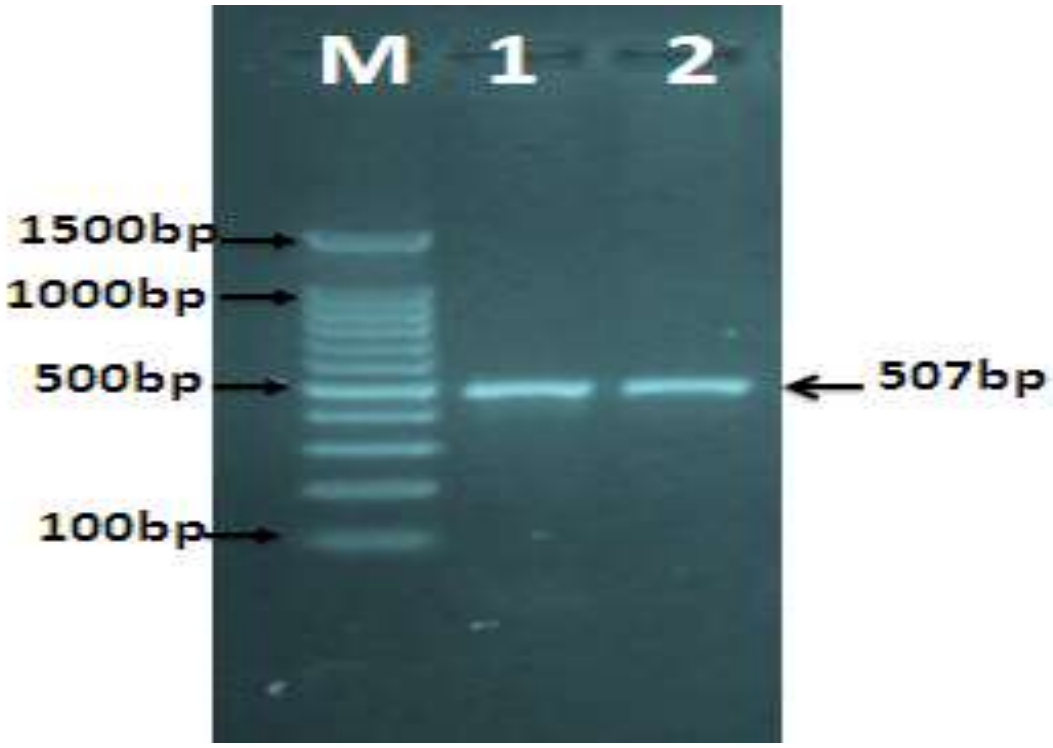

Figure 4.6 The PCR product analysis of the ropA gene in *Rhizobium etli* is shown in this agarose gel electrophoresis picture, where ladder (1500-100 bp), 1 and 2 represent the number of isolates indicated positive ropA gene at (507 bp) Size of the PCR product

A study by Koskey *et al.* (2018) and Pereira de Lyra *et al.* (2019) showed similar results, except for group 9, which consisted primarily of 1 or 2 isolates, and these isolates were made up of the three species of nodules, soils, and reference strains of *Rhizobium*. The inoculation test stations demonstrated mechanophilicity from prior research and the findings. Type of test plant utilized, symptoms, and virus or ethnicity isolated from plants, as well as a wide range by variety (Erdiller *et al.* 1997).

4.4 Bactericidal Activity

Rhizobium etli was shown to be sensitive to titanium dioxide nanoparticles, as illustrated in Figure 4.7. It was incubated in solutions of different concentrations of titanium dioxide nanoparticles (1250, 2500, and 5000 μg mL^{-1}) and cultured on yeast extract mannitol medium (YEM) against a control using the well diffusion agar technique (Holder and Boyce 1994). It can be seen in the figure that TiO_2 NPs did not have any pronounced toxic effect upon incubation with the nanomaterial, even at high concentrations. However, according to the visual observation, they, in turn, potentially demonstrated an increase in the growth rate in comparison to the control, as shown in the figure.

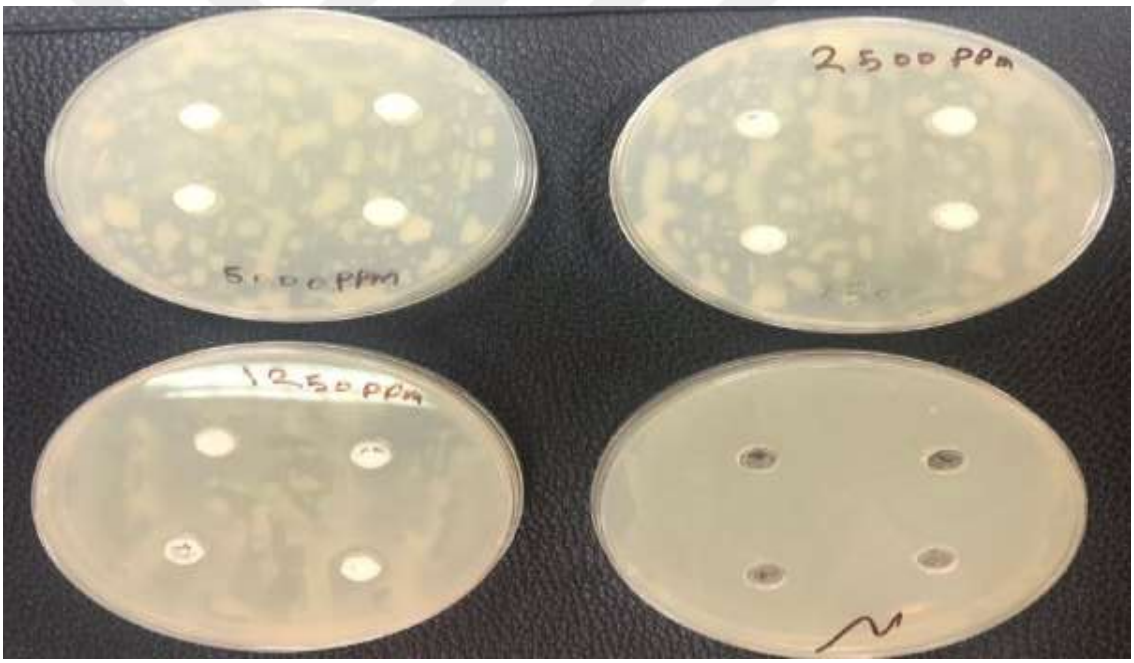

Figure 4.7 Biological activity of *Rhizobium etli* bacteria root nodule of broad beans plants

On the other hand, a study by Moll *et al.* (2016) stated that TiO_2 NPs (P25, E171) and non-nanomaterial of TiO_2 were utilized with the nitrogen fixing bacteria *R. trifoli* to investigate their effect on plant growth. TiO_2 NPs P25 did not show any remarkable impact on plant growth, but with reference to TiO_2 NPs E171 and non-nanoscale TiO_2, there seems to be a decline in the growth rate. More precisely, *R. trifoli*'s growth rate

was lowered by 43 and 23%,respectively, by E171 and non-nanoscale TiO_2, whereas P25 had no impact. The red clover release time was reduced by 41 to 62% for all TiO_2 NPs that were examined in this study. No nodules were identified in 21% of plants treated with TiO_2 NP. In hydroponic settings, certain TiO_2 NPs impede the development of *R. trifolii* and red clover, as well as their symbiotic relationship (Moll *et al.* 2016).

4.5 Molecular study of *Rhizobium etli* upon incubation with TiO_2 NPs

The RT-PCR amplification data are shown in Table 4.1, Figure 4.8, and Figure 4.9. Table 4.1 shows the gene expression before and after incubation of solutions of different concentrations of titanium dioxide nanoparticles (1250, 2500, and 5000 μg mL^{-1}) with *Rhizobium etli* in which each *in vitro* test was conducted using a randomized full-block design with three experimental blocks and three duplicates per block. Additionally, the gene expression of (ropA) gene in *Rhizobium etli* as depicted in Figure 4.,8 was studied for each incubated concentration of titanium dioxide nanoparticles with the bacteria and for the housekeeping gene, as stated in Figure 4.9.

Table 4.1 Statistical analysis of the gene expression results

Gene expression analysis						
No. sample	**CT (ropA)**	**CT (reC)**	**ΔCT Test**	**ΔCT Control**	**ΔΔCT**	**Fold change (2^-ΔCT)**
A: Negative control	34,18	30,10	4,08	3,51	0,57	0.672
	33,35	30,34	3,01	3,51	-0,50	1,411
	33,65	30,22	3,43	3,51	-0,08	1,055
B: 5000 ppm	31,08	30,20	0,88	3,51	-2,63	6,182
	32,10	30,83	1,27	3,51	-2,24	4,724
	30,62	30,10	0,52	3,51	-2,99	7,926
C:2500 ppm	33,66	31,16	2,50	3,51	-1,01	2,009
	32,88	30,31	2,57	3,51	-0,94	1,914
	32,64	30,23	2,41	3,51	-1,10	2,137
D:1250 ppm	32,88	31,20	1,68	3,51	-1,83	3,550
	34,19	30,14	4,05	3,51	0,54	0,686
	35,34	30,17	5,17	3,51	1,66	0,316

* The difference in gene expression fold change was due to the Real-time PCR gene expression experiments that depend on independent triplicate samples, and in every

experiment, the author used replicates to reduce the standard error. Besides, the author used the mean of sample replications (Figure Table 4.2).

Table 4.2 Standard deviation values for gene expression results

Descriptive Statistics		
	Mean	Std. Deviation
A: Negative control	1,5345	1,02254
B: 5000 ppm	5,7080	1,73491
C: 2500 ppm	2,0150	0,09192
D: 1250 ppm	1,8880	1,62427

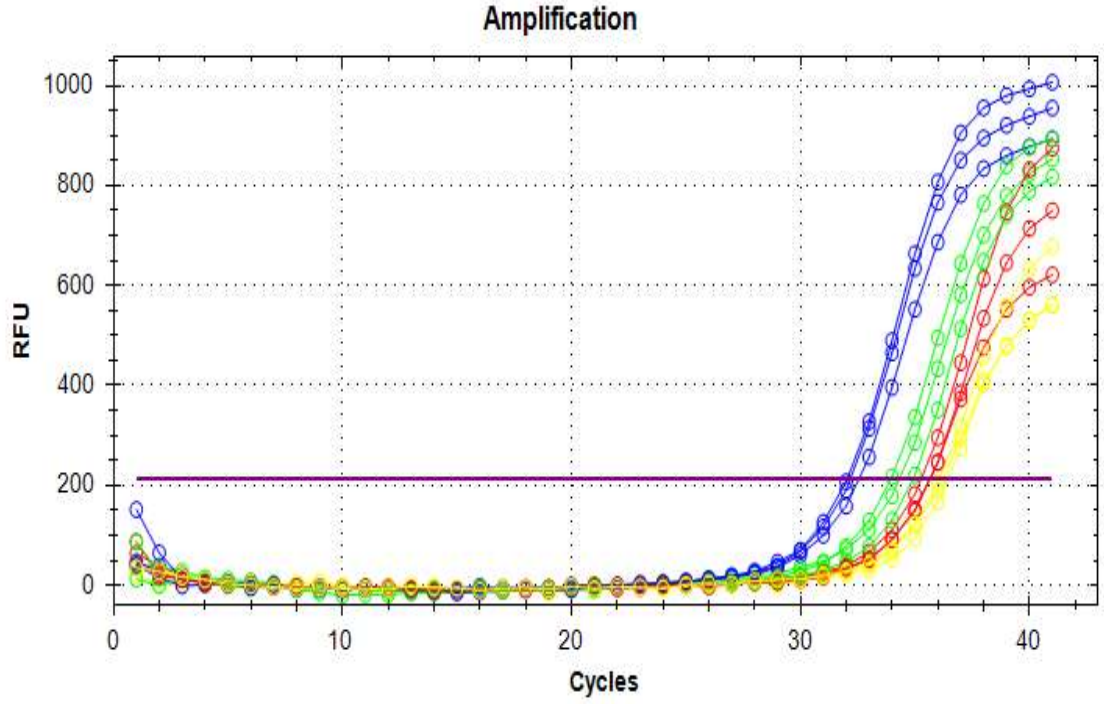

Figure 4.8 The results of the study sample amplification, the Real-Time PCR amplification plots of the target gene (ropA) in *Rhizobium etli* experimental isolates; where: A group is yellow plots, B group is blue plots, C group is green plots, D group is red plots.

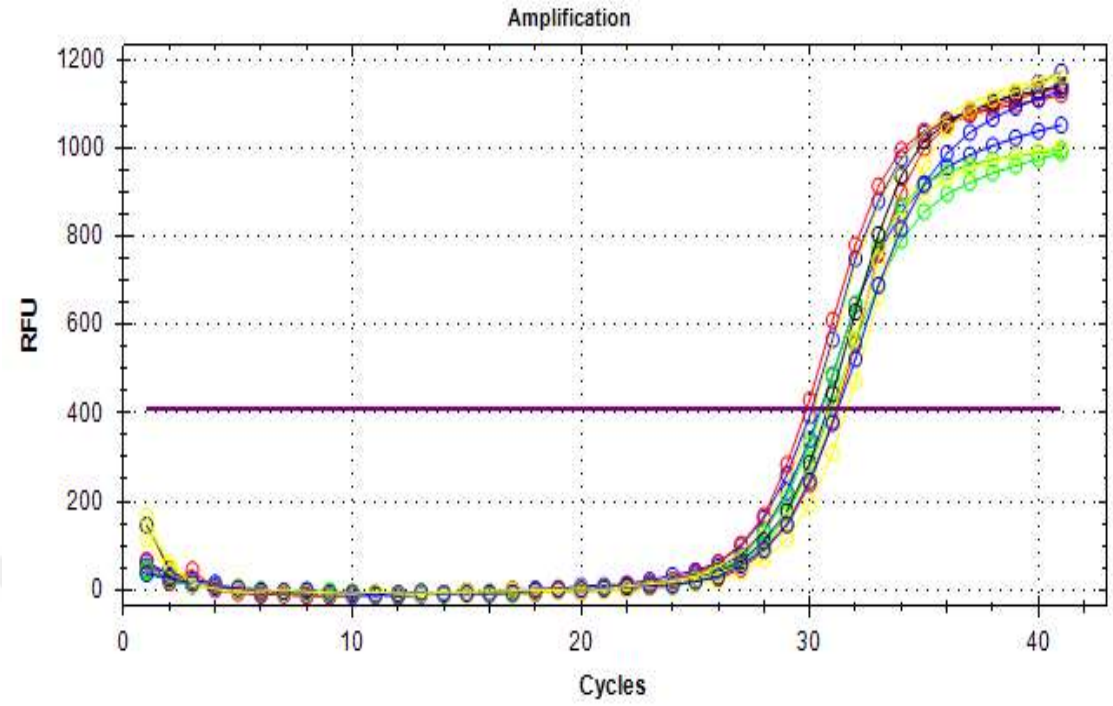

Figure 4.9 The results of the study sample amplification, the Real-Time PCR amplification plots of the house-keeping gene (recA) in *Rhizobium etli* experimental isolates; where: A group represents yellow plots, B group is blue plots, C group is green plots, and D group is red plots

***Threshold cycle line**: The real-time PCR threshold line is the level of signal that reflects a statistically significant increase over the calculated baseline signal; it is auto-set to distinguish relevant amplification signals from the background. The recA gene is a housekeeping gene used to compare the results of the ropA gene as an indicator of downregulation or upregulation that may happen when treating the bacteria with TiO_2 NPs. In addition, the housekeeping gene had fixed gene expression because it has a conserved nucleotide sequence.

As a result, the smallest fold change was found to be in the negative control with 1,046, followed by the highest average fold change of 6,277 for 5000 µg mL^{-1} of TiO_2 NPs, then, the mean fold change of 2,020 for 2500 µg mL^{-1} of TiO_2, and finally the mean fold change of 1250 µg mL^{-1} of TiO_2 NPs with 6,277. It can be seen in the table that as the concentration increases, the gene expression increases, which means that there are significant differences in gene expression at all concentrations that cause increases in

the growth rate of *Rhizobium etli* in comparison to the control sample of the bacteria. From the results obtained in this experiment, it was found that increases in the concentration of TiO_2 NPs also increased the growth of the bacteria (Figure 4.10). This is contrary to the other studies where titanium dioxide was suggested as a substance affected by light and oxygen that can produce free radicals that can affect the cell wall of bacteria (Chavan *et al.* 2020).

Therefore, the increase in the growth rate, if indicated, indicates the possibility of the presence of a plasmid inside the bacteria, which in turn gave the bacteria the ability to resist this substance and benefit from it in metabolism and division. Perhaps the best evidence for this is an increase in the rate of gene expression for the genes used after treating bacteria with different concentrations of TiO_2 NPs. This promotion in the growth rate was also explained by Mingfang *et al.* (2013) and may be attributed to the significant positive impact of TiO_2 NPs on photosynthesis and biosynthesis of photosynthetic pigments; the treatment of tomato seeds with TiO_2 NPs produced more energetic tomato plants in comparison with the untreated control. Moreover, the findings in this experiment are in line with that studied by Zheng *et al.* (2005) and Yang *et al.* (2007) who explained that TiO_2 NPs could cause more absorption of inorganic nutrients, improve the degradation of organo-compounds, and cause quenching of free radicals generated during the photosynthetic process, and as a result, enhance the photosynthetic efficacy.

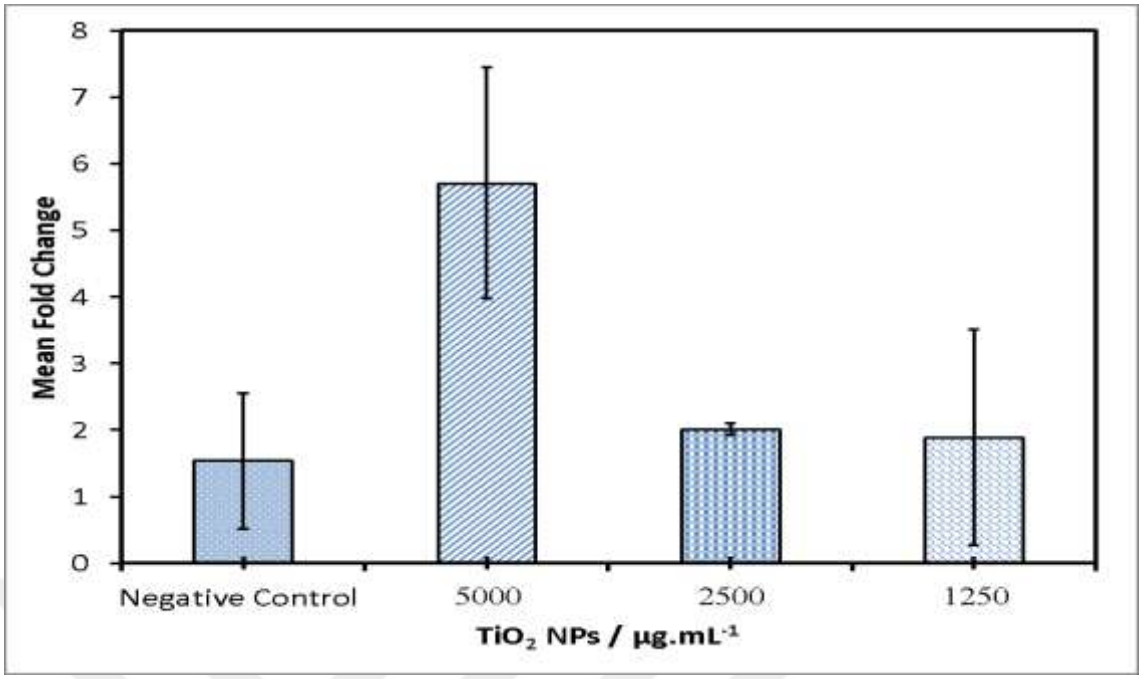

Figure 4.10 Statistical analysis of gene expression of *Rhizobium etli* bacteria before and after incubation with solutions of different concentrations of TiO_2 NPs (1250, 2500, and 5000 µg mL^{-1})

Another study by Simonin *et al.* (2016) agreed with the outcome of this study, which demonstrated that titanium dioxide was not toxic to *Rhizobium* bacteria when grown in the roots of bean daughters but was effective in increasing the growth of these bacteria; however, at higher nano-concentrations, there was increased growth due to higher N availability. TiO_2 NPs significantly affected soil microbial function by affecting trace nitrogen levels as well. This difference is likely because TiO_2 NPs reduced the effects of exposure on soil function, as measured by percentage, compared to controls. The investigation was done on a different type of bacteria than the previous one.

5. CONCLUSION AND RECOMMENDATION

5.1 Conclusion

it was explored a novel method for the improvement of growth of some Rhixobium app. through using titania nanoparticles. Here in, our study reported the successful synthesis of nano-sized titanium dioxide nanoparticles with a 25 nm particle size and a zeta potential of -37 mV. It was found in the roots of a bean plant and identified using the PCR method. With the use of Real-Time PCR, it was possible to compare the expression of the TiO_2NP-responsive (ropA) gene in *Rhizobium etli* to that of the housekeeping (rceA) gene in the same species after a 24-hour incubation period.

Rhizobium etli was found to grow more rapidly in the presence of TiO_2 NPs in this experiment. This research may be used as a blueprint for the production of nitrogen-fixing fertilizers that are more effective in promoting the development and activity of nitrogen-fixing bacteria after first testing *Rhizobium etli* with the plant and examining the link between the plant, bacteria, and titanium dioxide. It should also be evaluated in both lab and field studies.

5.2 Recommendations

In this section, we suggest some future works, which could be considered further. Therefore, we recommend these projects and they are as follows:

- ✓ The nanotoxicity of TiO_2 NP on other plant microorganisms and other strains of Rhizobium app. should be investigated.

- ✓ The impact of various nanomaterials on *Rhizobium* species should be investigated.

- ✓ Nanoparticle suspensions of TiO_2 may be applied directly to plants, or they can be irrigated into plant soil to study their in-situ effects on *Rhizobium* species.

- ✓ A molecular study of other genes of *Rhizobium etli* bacteria incubated with TiO_2 NPs should be conducted.

- ✓ The effect of particle size distribution and surface charge of TiO_2 NPs on *Rhizobium etli* bacteria should be studied.

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