DOCUMENTATION PAGE WITH ABSTRACT

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ABSTRACT

Catechol is considered to be a toxic organic compound and impacts dangerously on human heath and environment. Nowadays, phytoremedation has been proposed as a potential and cost-effective technology for treatment of hazardous environmental contaminants. And *Arabidopsis thaliana* is widely used in plant biology and genetic research areas, and has applied as a model plant in phytoremediation technique of organic chemical contaminants degradation. In this study, endophytic bacterium *Burkholderia cenocepacia* 869T2 was inoculated in the *Arabidopsis thaliana* plant to test ability to degrade Catechol. And *Achromobacter xylosoxidans* F3B train was chosen and cultured in the same Catechol contaminated environment with *Burkholderia cenocepacia* 869T2 to compare ability to grow and remove Catechol contaminants. The results showed that the *Burkholderia cenocepacia* 869T2 is unable to benefit growth of *Arabidopsis thaliana*, and cannot enhance phytoremediation of Catechol contamination as expected. Nevertheless, this study demonstrates that *Burkholderia cenocepacia* 869T2 can use Catechol as carbon source and grow well in the presence of Catechol pollutants. Moreover, this endophyte is extremely efficient to degradation of Catechol, even better than *Achromobacter xylosoxidans* F3B that had introduced in some previous researches of other researchers.

Keywords	1. Phytoremediation 2. Arabidopsis thaliana 3. Catechol 4. Endophyte 5. Burkholderia cenocepacia 869T2
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Sincerely,

Nguyen Thi Bao Anh

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1.	BCC	*Burkholderia Cepacia* Complex
2.	*B. cenocepacia* 869T2	*Burkholderia cenocepacia* 869T2
3.	*A. thaliana*	*Arabidopsis thaliana*
4.	*A. xylosoxidans* F3B	*Achromobacter xylosoxidans* F3B

PART I. INTRODUCTION

1.1. Research Rationale

As a developing country, Vietnam is progressing industrialization and modernization for the sustainable development while simultaneously promoting globalization with other nations in the world. Nevertheless, it is an undeniable fact that these processes bring lot of drawbacks, which release high concentrations of hazardous chemical compounds in the environment.

In some recent decades, “aromatic pollutant” is a familiar term and a concern of scientific areas and socio-economic one. Especially, through process of enzymatic conversion, aromatic compounds are degraded to Catechol by degradative bacteria. In most case, degradative bacteria convert enzymatically nonhalogenated aromatic compounds and xenobiotic chemicals, which are the main components of most pesticides, herbicides, refrigerants and solvents, to Catechol or protocatechuate. Hypothetically, Catechol is defined as an organic compound that is the ortho isomer of the three isomeric benzenediols (Chisholm and Hugh, 1911). In trace amounts of fruit and vegetable, it is an organic solid and colourless compound with the molecular formula $C_6H_4(OH)_2$ (Chisholm and Hugh, 1911). It is very rapidly soluble in water when being as feathery white crystals. With the fast solubility in water and other properties, Catechol is considered as a toxic compound that may cause risks and diseases to human health, and is easily distributed in the environment and causes soil and water pollution (Department of Health and Human Services, 1995). It is toxic by oral and dermal routes and harmful by inhalation, causes skin irritation, an allergic

skin reaction and serious eye damage. This compound also can mostly be founded in numerous sources, such as pesticides, flavors and fragrances, fruits, vegetables, tea, cigarette smoke, argan oil, etc. (Department of Health and Human Services, 1995). Therefore, this contaminant will become extremely serious risks of environmental and health issues.

Currently, numerous treatment methods have been applied to break down not only Catechol but also other toxic chemicals. However, those are either very expensive or not really effective. In this regard, phytoremediation is an advanced environmental-friendly and cost-effective technology for organic or inorganic pollutants treatment with various outstanding advantages (Punamiya et al., 2010; Saiyood et al., 2010), which uses green plants to stabilize or degrade contaminants to help mitigate recently environmental issues without influences on surrounding materials and dispose of it elsewhere (Environmental Protection Agency, 2011). Even though it takes a longer time for treatment, phytoremediation has considered to be more effective and beneficial than other methods. The inoculation of endophytic bacteria and phytoremediation plants has a better ability to degrade and neutralize the presence of compound pollutants with low level in soil and wastewater (Tesar et al., 2002; Chaudhry et al., 2005). Moreover, some other related researches in European countries and Taiwan have demonstrated that the combination of functional endophytic bacteria and plants can considerably reduce heavy metals, harmful organic compounds, etc., enhance the effectiveness of phytotoxicity and decrease the release of toxic volatiles into the atmosphere (Ho et al., 2013; Clay, K. and Schardl, C., 2002).

In this study, the model plant *Arabidopsis thaliana* will be inoculated with a functional endophytic bacterium *Burkholderia cenocepacia* 869T2 in the laboratory. The *Burkholderia cenocepacia* 869T2 are characterized with formable features in remediation of Catechol pollutants without harm to the *A. thaliana*, and even can protect against pollutant stress and benefit growth of the plant. *Burkholderia cenocepacia* 869T2 can utilize Catechol as sole carbon source for survival and degradation of Catechol. In addition, *Achromobacter xylosoxidans* F3B train was chosen and cultured in the same Catechol contaminated environment with *Burkholderia cenocepacia* 869T2 to compare ability to grow and remove Catechol contaminants. Therefore, this research will be a novel phytoremediation method to be significant and helpful for improving the efficiency of phytoremediation of Catechol pollutant removal and to bring a better effect for the plant growth.

1.2. Research's Objectives

(a) To utilize outstanding advantages of the inoculated *Arabidopsis thaliana* plant with *Burkholderia cenocepacia* 869T2 to degrade and remove Catechol pollutants.

(b) To strengthen ability of the *A. thaliana* against the presence of hazardous compounds Catechol and other organic contaminatants.

(c) To study ability of the endophytic bacteria *Burkholderia cenocepacia* 869T2 in growth and degradation of Catechol.

(d) To maintain and stimulate the plant growth by the inoculation with *B. cenocepacia* 869T2 better than that with other bacteria.

1.3. Research Questions and Hypotheses

With many advantages in the environmental and biological fields, the application of the Arabidopsis thaliana plant with *Burkholderia cenocepacia* 869T2 is a cost-effective measure to remove aromatic pollutants. And this scientific research will seek to answer the central research questions.

a) How do *A. thaliana* plants grow in contaminated environment by Catechol?

b) What are differences between inoculated *A. thaliana* and un-inoculated plants?

c) How does the functional endophytic bacterium *Burkholderia cenocepacia 869T2* benefit *Arabidopsis thaliana* plant?

d) How effective is *Burkholderia cenocepacia* 869T2 in degrading Catechol?

1.4. Limitation

The study has not bring positive result in the inoculation of *Arobidopsis thaliana* and the endophytic bacteria *Burkholderia cenocepacia* 869T2 and in improving phytoremediation of Catechol pollution.

PART II. LITERATURE REVIEW

2.1. Endophyte

2.1.1. Overview of Endophyte

Definition

An **endophyte** refers to an organism, often a bacterium or fungus, that lives within a host plant for without causing apparent disease and pathogens (Clay and Schardl, 2002; Carroll, 1986). Endophyte occurs within lichens and algae. In the natural environment, endophytes are ubiquitous and have been found widely and isolated from almost species of known plants (Faeth, 2009).

Functions

Many endophytic bacteria and fungi are capable to benefit the host phytoremediation plants by preventing colonization of pathogenic organisms and infection of diseases. Within plants, the endophytes colonize tissues and create a "barrier effect", where the local endophytes outcompete and prevent incidence of pathogenic organisms. Other interesting function is that the smart endophyte organisms can produce chemicals which inhibit the growth of their competitors. Additionally, endophytic species may promote host growth, and improve ability of plants against stresses and their resistance to mammalian herbivores and pest as well (Barac et al., 2004).

In terms of bioremediation, beneficial endophytes have been demonstrated to enhance the efficiency of phytoremediation and to reduce the severity of toxicity in plants species caused by contaminants (Ho et al., 2012, 2013).

Application

The endophytic organisms have various advantages and potential applications in many fields, such as bioremediation, medicine, industry and agriculture. The numerous varieties of compounds produced by endophytes are able to combat effectively pathogens and even cancers in animals and human beings. A concrete example of this is *Pestalotiopsis microspore* discovered by Gary Strobel, an endophytic fungus of *Taxus wallachiana* (Himalayan Yew) was detected to produce taxol which brought medicinal treatments to humans (Strobel et al., 1996). Furthermore, for agricultural usage, endophytes have been demonstrated to promote plant growth through the production of plant hormones, enhancement of nutrient uptake, nitrogen fixation, and biocontrol; increase crop yield such as barley, tomato, maize, etc. and enhance tolerance against many root-pathogens and diseases (Varma et al., 1999; Waller et al., 2005).

Until now, there are thousands of endophytic species which are helpful to mankind, but many endophytes might be permanently lost before their utility is explored because of deforestation, biodiversity loss, climate changes, etc. At the present, the effects of climate change on endophytes is being investigated, and scientific researches of plants grown at different climates or at increased carbon dioxide levels have different distributions of endophytic species.

2.1.2. Introduction to Burkholderia cenocepacia 869T2

Burkholderia cepacia complex (BCC), or simply *Burkholderia cepacia*, naturally found in the environment, consists of a group of catalase-producing, lactose-

nonfermenting and Gram-negative bacteria. They typically exist in water and soil, and can survive for prolonged periods in moist environments. There are about 18 different species, namely *B. cepacia, B. multivorans, B. cenocepacia, B. vietnamiensis, B. stabilis, B. ambifaria, B. dolosa, B. anthina, B. pyrrocinia and B. ubonensis* (Lipuma, 2005).

Furthermore, *Burkholderia cenocepacia*, a member of BCC, is ubiquitous in the environment and may cause disease in plants. However, *Burkholderia cenocepacia* 869T2 bacterium is, a new strain of *B. cenocepacia*, was successfully isolated from vetiver roots by Chieh-Chen Huang and his collaborators in 2014, which is strongly antagonistic against *Fusarium oxysporum f. sp. cubense tropical race 4* (Foc TR4) and significant potential for use in a good biocontrol agent of Fusarium wilt and in promoting growth in banana plants (Ho et al., 2014). The *B. cenocepacia* 869T2 is only isolated from vetiver grass and has demonstrate that this strain has potentials as both bio-controller and bio-fertilizer. In some previous researches, *B. cenocepacia* showed the ability to colonize the surface of hyphae of *F. oxysporum f. sp. cubense* (Panet et al., 1997). Endophytic bacteria, such as Burkholderia sp., have been investigated as biocontrol agents for plantlets of banana "Maça." (Weber et al., 2007), vine plantlet protection (Kilani-Feki and Jaoua, 2011), and date palm seedlings (Dihazi et al., 2012).

Currently, researchers in the world are working to learn further about characteristics and study applications of this endophyte. This new strain *Burkholderia cenocepacia* 869T2 bacterium is worthy and promising for applied sciences in general and bio-control in particular in future.

2.1.3. Introduction to Achromobacter xylosoxidans F3B

Achromobacter xylosoxidans is an aerobic, Gram-negative, rod oxidase- and catalase-positive bacterium with peritrichous flagella for motion (Igra-Siegman, Chmel and Cobbs, 1980). It was first described by Holmes et al, and named and characterized officially by Yabuuchi and Ohyama in 1971. It was isolated from patients with pulmonaryand belongs to the genus *Achromobacter* found in water environments. This species can cause infections like bacteremia, especially for patients who suffer from cystic fibrosis. Several previous studies have proven capability of *A. Xylosoxidans* in removal of Catechol and monoaromatic hydrocarbons (BTEX) (Moon et al., 1997; Nielsen et al., 2006), and the strain F3B can help inoculated *Arabidopsis thaliana* plant and vetiver grass to tolerate stress from aromatic compounds and to improve phytoremediation of phenolic pollutants. Up to now, this functional endophytic bacterial strain is being studied for more potential applications.

2.2. Phytoremediation

Definition

In recent years, phytoremediation is an eco-friendly and advanced technology for sciences, which uses directly phytoremediation plants and the combination with endophytes to degrade or remove environmental pollutants in soils, sludges, sediments, surface water or ground water (Environmental Protection Agency, 2011). It is effectively applied in treating low levels of organic or inorganic contaminants from polluted sites without influences on surrounding materials and dispose of it elsewhere. Even though it takes a longer time for treatment, phytoremediation has considered to be more effective and beneficial than other methods.

Processes of Phytoremediation

The phytoremediation of metal or inorganic compounds mediated by plants or algae useful in treatment of environmental issues (Glick, Pasternak and Patten, 2009), including

- *Phytoextraction* is a process of absorption and concentration of chemicals from the soil into roots and shoots of the plant.

- *Rhizofiltration* is a bioremediation process which use plant roots to remove metals from effluents.

- *Phytostabilization* is a process stabilization of substances in the environment, which prevent the spread of metals in the environment

The phytoremediation of organic compounds may occur by phytostabilization, i.e., reducing the spread of organic material in the environment.

- *Phytotransformation* refers to chemical modification of environmental substances as a direct result of plant metabolism, often resulting in their inactivation, phytodegradation, or phytostabilization.

- *Phytostimulation* is the stimulation of activity of soilborne microbes, especially root-microbial organisms, for removal ofcontaminants.

The process of uptaking contaminants in plants mostly takes place through the root system, in which the principal mechanisms for preventing contaminant toxicity are found. The utility of the use of trees with long root system is more effective in treating deeper contamination because tree roots penetrate more deeply into the ground. Besides, plant roots impact on the population and activity of the microorganisms, the aggregation and stability of the soil particles and chemical subtances around the root.

Application

Phytoremediation may be applied worldwide in treatment of environmental issues, especially focus on contaminated soil and water, contaminants may be heavy metals, organic compounds, pesticides, solvents, explosives (Chisholm and Hugh, 1911), and crude oil and its derivatives. Many plants such as vetiver grass, Arabidopsis thaliana, mustard plants, alpine pennycress, hemp, and pigweed have proven to be successful remove toxic chemicals or compounds.

Recently, the inoculation of endophytic bacteria and phytoremediation plants has a better ability to degrade and neutralize compound pollutants' presence with low level in soil and wastewater. Furthermore, the combination of phytoremediation plants with beneficial endophytic bacteria to promote degradation or removal of toxic compounds has been proposed recently (Environmental Protection Agency, 2011; Tesar et al., 2002), and it seems to be a potential tool to reduce phytotoxicity, increase the severity of pollutants in soil, water and the atmosphere as well.

2.3. Plant-microbe phytoremediation

Phytoremediation is effective in eliminating low concentration of pollutants from contaminated sites. Although using plants for remediation of persistent organic compounds pollutant brings benefits than other methods, there are still some limitations. For example, in the presence of very high concentration of contaminants, some plants grow slowly and are quite small. To remedy this issue, plant-microbe phytoremediation is considered as an advanced approach to enhance the efficiency of phytoremediation, i.e. functional endophytic bacteria are inoculated with phytoremediation plants. In this case, functional endophytic bacteria have been used to

reduce organic chemical contaminants (Germaine et al., 2006) and can help the plant tolerate lethal concentrations of aromatic compounds (Yo et al., 2013). The inoculation of plants with functional endophytic bacteria is useful to reduce effects of phytotoxity in plants, benefit plant growth, improve efficiency of pollutant removal, and reduce release of volatile compounds into the atmosphere (Barac et al., 2004). Therefore, plant-microbe phytoremediation is a promising and improved technology to deal with both organic and inorganic environmental contamination

2.4. Overview of Arabidopsis thaliana

Being discovered in the Harz Mountains in the sixteen century by Johannes, *Arabidopsis thaliana* is defined as a Eurasian small flowering plant, which is belonged to the mustard (Brassicaceae) family. In the 1900s, the first study about this plant was carried out, and *Arabidopsis thaliana* was proposed as a model organism for genetics in 1943 by Friedrich Laibach (Yanofsky et al., 1990).

Figure 2.1: View of Arabidopsis thaliana.

(source:https://www.arabidopsis.org/portals/education/aboutarabidopsis.jsp)

The *Arabidopsis thaliana* possesses a very small genome of approximately 125 Mb which is 19 times as mall as corn plant's and 128 times as mall as wheat's, thereby it is an ideal and significant tool for understanding the molecular biology of many plant traits (Dennis, 2004; Mysore, Tuori and Martin, 2001). Moreover, the plant is well-known as an annual plant with a relatively short life cycle (about six weeks). In a period of lifetime, the plant can be around 20 cm tall and its flower has nature of self-pollination that is helpful for genetic experiments. With a small size and rapid lifecycle, *Arabidopsis thaliana* plant is conveniently grown in a small space, for example pots or Petri plates in the suitable condition of fluorescent lights in labrary or in a greenhouse, and produces several thousand seeds in each individual for doing experiment in labratory. Therefore, the *A. thaliana* plant has brought lot of benefits for science, and nowadays, is widely applied as a model organism for researches on plant genetic, cellular and molecular biology as mice and fruit flies (*Drosophila*) play in animal biology (Rensink and Buell, 2004; Coelho et al., 2007).

2.5. Catechol

2.5.1. Definition

Catechol is defined as an organic compound that is the ortho isomer of the three isomeric benzenediols (Chisholm and Hugh, 1911). In trace amounts of fruit and vegetable, it is an organic solid and colourless compound with the molecular formula $C_6H_4(OH)_2$. This chemical has many alternative names, such as 1,2-Benzenediol; pyrocatechol; o-dihydroxybenzene; pyrocatechin; o-diphenol; Durafur Developer C; o-hydroquinone; o-hydroxyphenol; oxyphenic acid; Pelagol Grey C; o-phenylenediol; pyrocatechinic acid. Nowadays, Catechol is commonly called "Pyrocatechol".

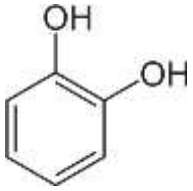


Figure 2.2. The molecular structure of Catechol

(Source: http://en.wikipedia.org/wiki/Catechol)

Furthermore, Catechol is well-known a product of the enzymatic conversion process of aromatic compounds by degradative bacteria. In most case, degradative bacteria convert enzymatically nonhalogenated aromatic compounds and xenobiotic (unnatural," or synthetic; from the Greek *xenos*, meaning "foreign") chemicals, which are the main components of most pesticides, herbicides, refrigerants and solvents, to Catechol or protocatechuate (Glick, Pasternak and Patten, 2009).

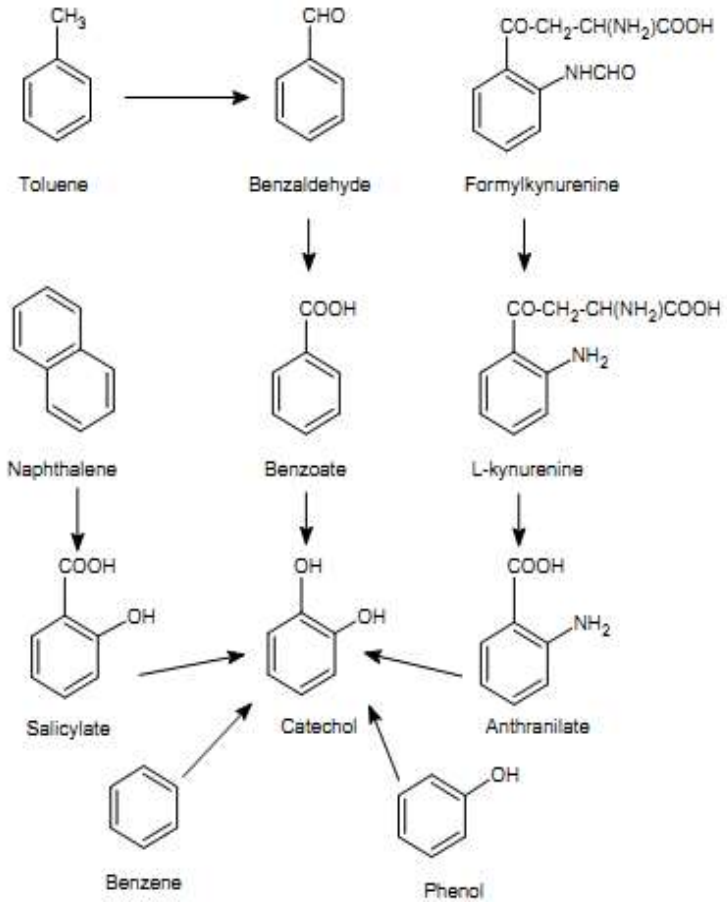


Figure 2.3. Pathways for the enzymatic conversion of aromatic compounds to Catechol by degradative bacteria (Glick, Pasternak and Patten, 2009).

2.5.2. Chemical and Physical Properties

Catechol was first discovered by destructive distillation of catechin extracted from plant, and in 1839 H. Reinsch isolated Catechol sucessfully by distilling it from the solid tannic preparation catechin. It is well-known as one of a hazardous aromatic compounds, and it is very rapidly soluble in water when being as feathery white crystals (Chisholm and Hugh, 1911; Department of Health and Human Services, 1995).

Table 2.1. Chemical and physical properties of Catechol (Department of Health and Human Services, 1995).

Property	Value
Physical state	Crystalline solid flakes at 20°C and atmospheric pressure
Colour	White to beige-brown
Odour	Slight phenol-like
Molecular weight	110.1 g/mol
Melting Point	105°C at atmospheric pressure
Boiling Range	245.5 °C at atmospheric pressure
Flash point	127°C (closed cup) at atmospheric pressure (value measured on the molten solid)
Flammability	Non flammable
Explosive properties	Non explosive
Self-ignition temperature	510°C at atmospheric pressure
Vapour pressure	0.05 hPa at 25°C, low volatility
Water solubility	517.5 g/l at 20°C, very soluble in water
Octanol Water partition coefficient (log Kow)	0.93 at room temperature low potential for bioaccumulation

2.5.3. Sources

In nature, Catechol is found widely in the component in products, such as fruits, vegetables and tea. In tea, catechol is as a skeleton structure in catechin. It is also one of the main natural phenols in argan oil, and its presence has been detected in *Agaricus bisporus*, castoreum and cigarette smoke.

Moreover, as a precursor in industry of producing pesticides, flavors and fragrances, Catechol is used as a photographic developer, a developer for fur dyes, as an intermediate for antioxidants in rubber and lubricating oils, in polymerization inhibitors, and in pharmaceuticals. Catechol may be released into the environment during its manufacture and use, and a significant 20 million kg of Catechol is reportedly produced each year. And the consumption of contaminated drinking water and ingestion of contaminated food are main routes of human exposure to and contamination of Catechol.

2.5.4. Impacts of Catechol

2.5.4.1. Impacts on Human and Animal Heath

With the fast solubility in water and other properties, pure Catechol is toxic and has antioxidant properties that may cause risks and diseases to human health.

In humans, catechol is an eye and skin irritant, a skin sensitizer and a depressant of the central nervous system (CNS). It also results eye burn that slow to heal (Department of Health and Human Services, 1995). Skin contact causes an eczematous dermatitis in patients. In addition, the absorption of this toxic chemical through the skin has caused illness resembling those of phenol poisoning, expect that the Catechol induced convulsions are more severe than those caused by phenol poisoning (Department of

Health and Human Services, 1995). With a high concentration, Catechol can depress the CNS and a prolonged rise of blood pressure in animals. The blood pressure is increased to be because of peripheral vasoconstriction. The International Agency for Research on Cancer (IARC) has classified catechol as a Group 2B, possible human carcinogen.Besides, the results of acute animal tests in rats, mice, guinea pigs, and rabbits have demonstrated catechol to have high acute toxicity by oral or dermal exposure.

2.5.4.2. Impacts on the Environment

Based on its physical and chemical properties, especially, it is very rapidly soluble in water, catechol is easily distributed in the environment and causes soil and water pollution (Department of Health and Human Services, 1995).

The potential of distribution in the atmosphere is very low and if catechol was released into the air, it would be readily degraded, so no significant air exposure is expected. However, the industrial productions and consumptions of Catechol or products of Catechol release the substance that directly wastes water sources (lake, reservoir, stream, etc.) (Chisholm and Hugh, 1911).

2.6. Researches on Phytoremediation Technology

2.6.1. International Researches

The concept of using plants to deal with environmental pollution is not new, nevertheless, this in situ remediation technology, that based on the concept of using nature to cleanse nature, takes advantanges and potentials of living organisms, namely plants and endophytes. Up to now, phytoremediation has received lots of concerns and inspired environmetnalists and other scientists to find out new ecologically friendly approaches for the environment and biodiversity.

About 300 years ago, plants were proposed for the treatment of wastewater (Hartman, 1975). At the end of the 19th century, *Thlaspi caerulescens* and *Viola calaminaria* were the first plant species used to accumulate high levels of metals in leaves (Baumann, 1885). Using plants to remove metals from contaminated soil was reintroduced and developed by Utsunamyia in 1980 and by Chaney in 1983, and the field trial on Zn and Cd phytoextraction was first implemented in 1991.

Over the past 20 years, this technology has become increasingly popular and has been employed at sites with soils contaminated with heavy metals and toxic compounds. Today many researchers, institutes and companies are made efforts to test different plants' effectiveness at removing a wide range of contaminants.

In North America, phytoremediation of petroleum hydrocacbons in soil is conducted to determine efficacy of agricultural and non-crop plants for degradation of aged petroleum hydrocarbons in soil at multiple locations and under varied climatic conditions in 1999. Additionally, in the May 1999 scientists from the UK reported that transgenic tobacco plants can play a role in cleaning up explosives.

Other studies showed that vetiver grass could accumulate and remove heavy metals such as lead, cadmium, copper, zinc and arsenic (Singhakant et al., 2009); and *Achromobacter xylosoxidans* could degrade catechol and monoaromatic hydrocarbons (BTEX) (Moon et al., 1997; Nielsen et al., 2006).

In Taiwan, Chieh-Chen Huang and other researchers in Department of Life Sciences, National Chung Hsing University had conducted successfully researches about “the combination of vetiver grass with a functional endophytic bacterium, *Achromobacter*

xylosoxidans F3B, for aromatic pollutants removal" in 2012 and "Selection and application of endophytic bacterium *Achromobacter xylosoxidans* strain F3B for improving phytoremediation of phenolic pollutants" in 2013. And his newest study, in October 2014, is application of *Burkholderia cenocepacia* 869T2 inoculated in banana plants to decreased Panama disease incidence of Fusarium wilt and improve the growth of banana plants (Ho et al., 2012, 2013 and 2014). The above-mentioned researches have demonstrated that the combination of functional endophytic bacteria and plants can considerably eliminate heavy metals, harmful organic compounds, etc., enhance the effectiveness of phytotoxicity, decrease the release of toxic volatiles into the atmosphere and stimulate growth of plants.

2.6.2. Researches in Vietnam

Phytoremediation has been studied and applied research area in Vietnam. For example, Vo Van Minh in Hanoi University of Natural Science studied on the Absorption of Heavy Metals in the Soil by Vetiver Grass and Assessment of its Effectiveness in the Rehabilitation of Contaminated Lands. And the research "Phytoremediation potential of indigenous plants from Thai Nguyen province, Vietnam" clarified the potential of six these plants as good candidates for phytoremediation of heavy metal pollution soil are being carried out in our laboratory.(Anh et al., 2011).

In conclusion, the mentioned-above literature review has covered basic and essential knowledge and the related researches about phytoremediation technology and beneficial endophytic bacteria. The combination of known phytoremediation plants and beneficial endophytic bacterium in improving phytoremediation of contaminants

is a new approach. Moreover, the *Burkholderia cenocepacia* strain 869T2 is recently discovered, thereby it is a new product of biotechnology and needed to be exploited more its applications in scientific areas. Therefore, this is the first report to use *Burkholderia cenocepacia* 869T2 with *A. thaliana* plants to take advantages in degradation of Catechol pollutants. It is evident that the benefits brought by this technology are various and valuable, and this study is a breakthrough for treating not only Catechol but other hazardous organic compounds.

PART III. METHODS

In each step of the study, different materials and equipment will be used for specific purposes and functions.

3.1. Materials

- ***For creating ½ MS Medium for Arabidopsis thaliana:***

- Murashige and Skoog Basal Medium.

- 3% sucrose; 0.8% agar; KOH (pH = 5.7), 500 ml of pure water.

- ***For sterilization of the sample seeds:***

- 70% EtOH; 10% Bleach; sterile H_2O

- *Arabidopsis thaliana* seeds (sample).

- ***For inoculation experiments and Catechol toxicity:***

- *Burkholderia cenocepacia 869T2*

- *Achromobacter xylosoxidans F3B*

- M9 Medium (Aseptically add the following sterile solutions: 1M MgSO4, 2 ml; 1M CaCl2, 0.1 ml)

- Catechol

- 70% EtOH

3.2. Equipment

- Pipetmans

- Microcentrifuge Tubes

- Sterile tips

- Timer

- Plates

- Clean Bench
- 3D Micro Shaker
- Mettler Toledo
- GeneQuant 1300 Spectrophotometer
- Hitachi centrifuge
- LM-570RD Shaker Incubator
- Fuego SCS Basic Laboratory Gas Burner
- Jasco HPLC system
- Inertsil ODS-2 HPLC Column
- 254 nm UV/VIS detector.

3.3. Methods

3.3.1. Creation of ½ MS Medium for Arabidopsis thaliana

1.11g of Murashige and skoog Basal Medium, 3% sucrose, and 0.8% agar were added together in a tube, and were mixed well with 500 ml of pure water to become a medium. After that, using Mettler Toledo, the medium was measured and adjusted carefully to pH of 5.7 with KOH. If the pH is too high, HCl is a good chemical substance to reduce pH level. When the medium's pH was reached to a needed level, it was next sterilized by autoclave for 20 mins. The sterilized medium was poured into plates, and waited for being cooled.

3.3.2. Surface sterilization of Arabidopsis thaliana Seeds

Arabidopsis thaliana seeds are put in an Eppendorf tube, and then 70% EtOH was added into the tube and suspended by turning the tube for 1min. For doing bleach treatment, the EtOH solution was poured out and 10% bleach was added and shaken

gradually by 3D Micro Shaker. After 20mins of shaking, the bleach was completely discarded, because the remain bleach can poison seedlings. All seeds continued to be thoroughly washed in a large amount of sterile water, and then the sterile water was discarded. This step was repeated for 3 or 4 times.

3.3.3. Cultivation of Sterilized Arabidopsis thaliana Seeds

The seeds were cultivated into 4 plates which contain the medium prepared before in enough spaces to grow well. And then they were lived and grown in a growth chamber at a constant temperature of 22 ^{o}C with 14/10 h light and dark cycle.

3.3.3.1. Preparation of Bacteria

50 µl of *Burkholderia cenocepacia* 869T2 and 50 µl of *Achromobacter xylosoxidans* F3B were taken and cultured into 2 tubes of 5 ml LB. After incubation period of for six hours at 30 ^{0}C, 200 r.p.m to an approximate OD600nm value of 0.57 and 0.98 for *A. xylosoxidans* F3B and *B. cenocepacia* 869T2 respectively, fresh endophytes were diluted one hundred-fold in water.

3.3.3.2. Inoculation of Arabidopsis thaliana with Endophytic Bacteria

Seven-day-old *Arabidopsis thaliana plants* were immersed in bacteria for 1min, and then washed by sterile water before being immersed quickly into 70% EtOH. The one-week-old plants, include inoculated plants that were inoculated with *Burkholderia cenocepacia* 869T2, and non-inoculated plants or control plants which were not inoculated, were transferred onto treatment plates which contain 1/2 MS agar mixing with three different concentrations of Catechol (0.2 mM, 0.8 mM and 1.6 mM). And then all plates were grown in a growth chamber at a constant temperature of 22 ^{0}C with 14/10 h light and dark cycle. After a period of seven days, growth parameters of

inoculated plants and non-inoculated ones, namely fresh weight and root length, were measured. Three replicas were carried out for each experiment.

3.3.4. Characterization of Endophytic Bacteria Strains

The ability of the bacterial endophytes to degrade Catechol and to survive in the Catechol pollutant environment were assessed by their growth in the presence of Catechol. In order to compare with other strains, *Achromobacter xylosoxidans* F3B strain was chosen. Both *Burkholderia cenocepacia* 869T2 and *Achromobacter xylosoxidans* F3B bacteria, that were incubated for six hours in the 1.4.1 step, were centrifuged at 12000 r.p.m for 1min and spread over M9 agar (Aseptically add the following sterile solutions: 1M MgSO4, 2 ml; 1M CaCl2, 0.1 ml) plates, which contain three different concentrations of Catechol (0.2 mM, 0.8 mM and 1.6 mM), and incubated for a week at 30 ^{o}C. No other carbon source was included in the medium.

3.3.5. Catechol Removal Test

Similarly to the step 3.4, *Burkholderia cenocepacia* 869T2 and *Achromobacter xylosoxidans* F3B bacteria were cultured into tubes which each tube contained 10 ml of M9 medium and 1.6 mM Catechol. For the purpose of comparison, 1.6 mM Catechol is added into the M9 medium tubes, which were used as a control (without bacteria). The controls (without bacteria) and the bacteria, in Catechol environment, were received the same treatment (at 30 ^{0}C and 200r.p.m) for 3days in a LM-570RD Shaker Incubator. 1.5ml samples are taken from the medium every 24h. There are three replicas for each sample taken every day. The samples will be directly analyzed with a Jasco HPLC system using an Inertsil ODS-2 HPLC Column provided with a 254 nm UV/VIS detector.

3.3.6. Imaging and Analysis.

The plants after seven-day growth and seven-day inoculation with Catechol toxicity were carefully measured the root length and fresh weight of all inoculated plants and un-inoculated ones**.** Remaining Catechol amount in the medium with *Burkholderia cenocepacia* 869T2 and *Achromobacter xylosoxidans* F3B bacteria was determined, and samples of the endophytic bacteria were taken photos. And the results were analyzed and compared.

3.3.7. Statistical Analysis

After analysis sample, available statistics and collected data were measured and analyzed. All statistical data were assessed in tabular form, figures and graphs to indicate differences between plants inoculated with *Burkholderia cenocepacia* 869T2 and the control plants in various concentrations of the toxic compounds Catechol, and the impacts of *Burkholderia cenocepacia* 869T2 and another strain in Catechol removal.

PART IV. RESULTS

4.1. The Effect of Catechol Toxicity on Plant Growth.

- ***Uninoculated plants***

After seven-day exposure to Catechol toxicity, the average root length and fresh weight were measured. The figure 4.1 shows that the uninoculated control plants (0.0 mM catechol and without *Burkholderia cenocepacia* 869T2) grew better than the plants induced by Catechol pollutants. For the uninoculated plants in the presence of catechol, the higher concentrations of Catechol were, the worse the growth of the uninoculated plants was. The uninoculated plants induced by 0.2 mM Catechol had more and longer roots, and developed healthier than the uninoculated ones exposed to 0.8 mM and 1.6 mM Catechol, as shown in the figure 4.1.

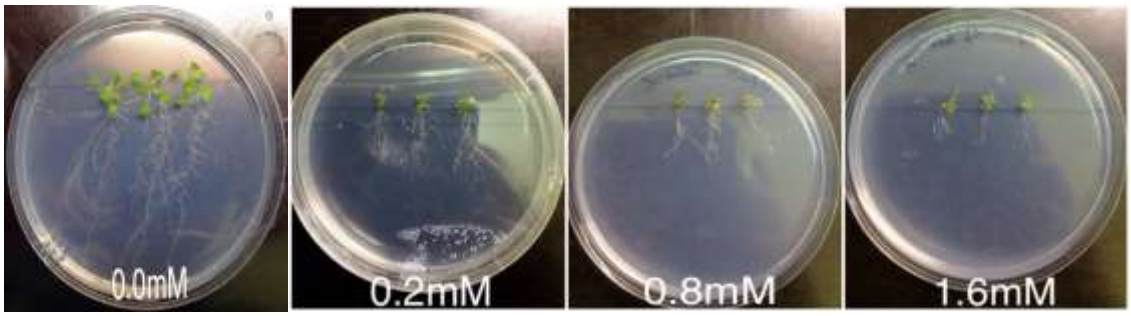


Figure 4.1. The uninoculated (control) plants on agar after seven-day exposure to different Catechol concentrations.

Table 4.1. The average root length with at least three uninoculated plants in each plate after a seven-day exposure.

	0.0 mM Catechol	**0.2 mM Catechol**	**0.8mM Catechol**	**1.6mM Catechol**
Root length (cm)	5.29±1.12	3.17 ±0.74	2.37±0.45	2.03±0.12

Table 4.2. The average fresh weight with at least three uninoculated plants in each plate after a seven-day exposure.

	0.0 mM Catechol	0.2 mM Catechol	0.8mM Catechol	1.6mM Catechol
Fresh Weight (mg)	10.66±3.12	3.9±0.46	4.3±0.38	4.2±0.44

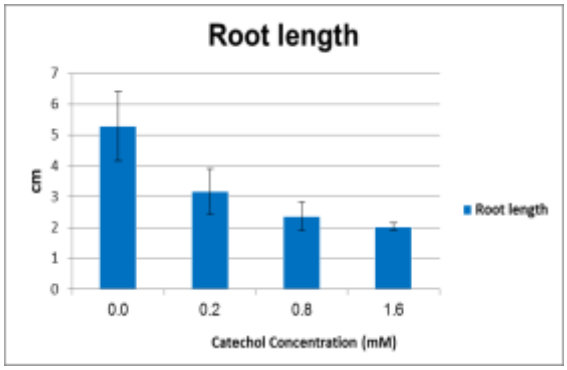


Figure 4.2. The average of root length with at least three plants in each plate after a seven-day exposure.

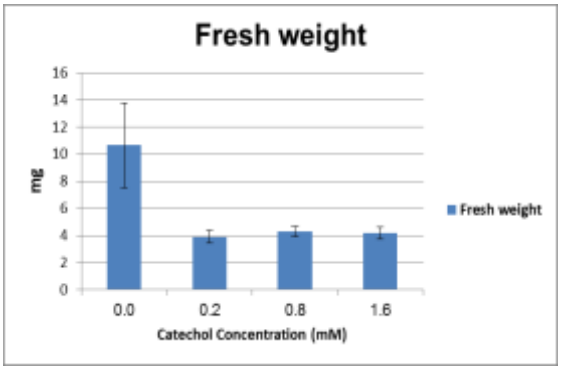
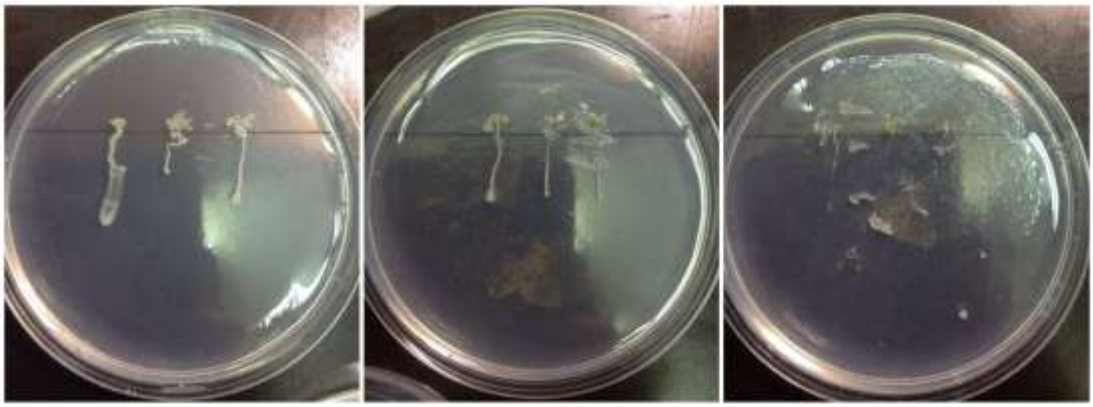


Figure 4.3. The average of fresh weight with at least three plants in each plate after a seven-day exposure.

As can be seen from the graph 4.1 and table 4.1 and table 4.2, it is clear that the average root length and fresh weight of the plants were decreased following to the increase of Catechol toxicity. The uninoculated plants contaminated and grown in Catechol from 0.2 mM to 1.6 mM Catechol had the steady decline in root length and fresh weight. As shown on both table 4.1 and 4.2, it is notable that the plants impacted by 1.6 mM Catechol had the shortest of root length and slightest of fresh weight. And there was a significant difference in the average root length of the the uninoculated plants induced by 0.2 mM Catechol and 1.6 mM Catechol.

For inoculated plants



0.2mM **0.8mM** **1.6mM**

Figure 4.4. The inoculated plants on agar after seven-day exposure to different Catechol concentrations.

The plants inoculated with *Burkholderia cenocepacia* 869T2 were colonized by bacteria and died. In contrast to the hypothesis assumed, this endophytic bacteria strain did not benefit the inoculated plants, the plants could not grow under effects of this train and Catechol pollutants. The figure 4.4 indicated the unexpected results of the worst of plant growth by impacts of the *B. cenocepacia* 869T2 train and Catechol toxicity.

4.2. The Growth of Bacteria in Catecol Toxicity

Two *Burkholderia cenocepacia* 869T2 and *Achromobacter xylosoxidans* F3B strains already received the same treatment during a seven-day period of incubation with no carbon source. The figure 4.5 reveals that both of these endophytic bacteria could utilized Catechol as a carbon source and developed in the Catechol contaminated condition. As can be seen from the figures, it is evident that *B. cenocepacia* 869T2 and *A. xylosoxidans* F3B grew well along a week, the growth of bacteria in the 7th day was better and colonies of bacteria were more than those in the 3rd day.

Figure 4.5. The growth of Burkholderia cenocepacia 869T2 in the 3rd day and 7th day of Catechol exposure.

Nevertheless, the increasing of Catechol levels resulted the increasing of contamination or the decline of bacteria growth. The *Burkholderia cenocepacia* 869T2 contaminated by 0.2 mM grew better and had more colonies than bacteria in 1.6 mM Catechol plate.

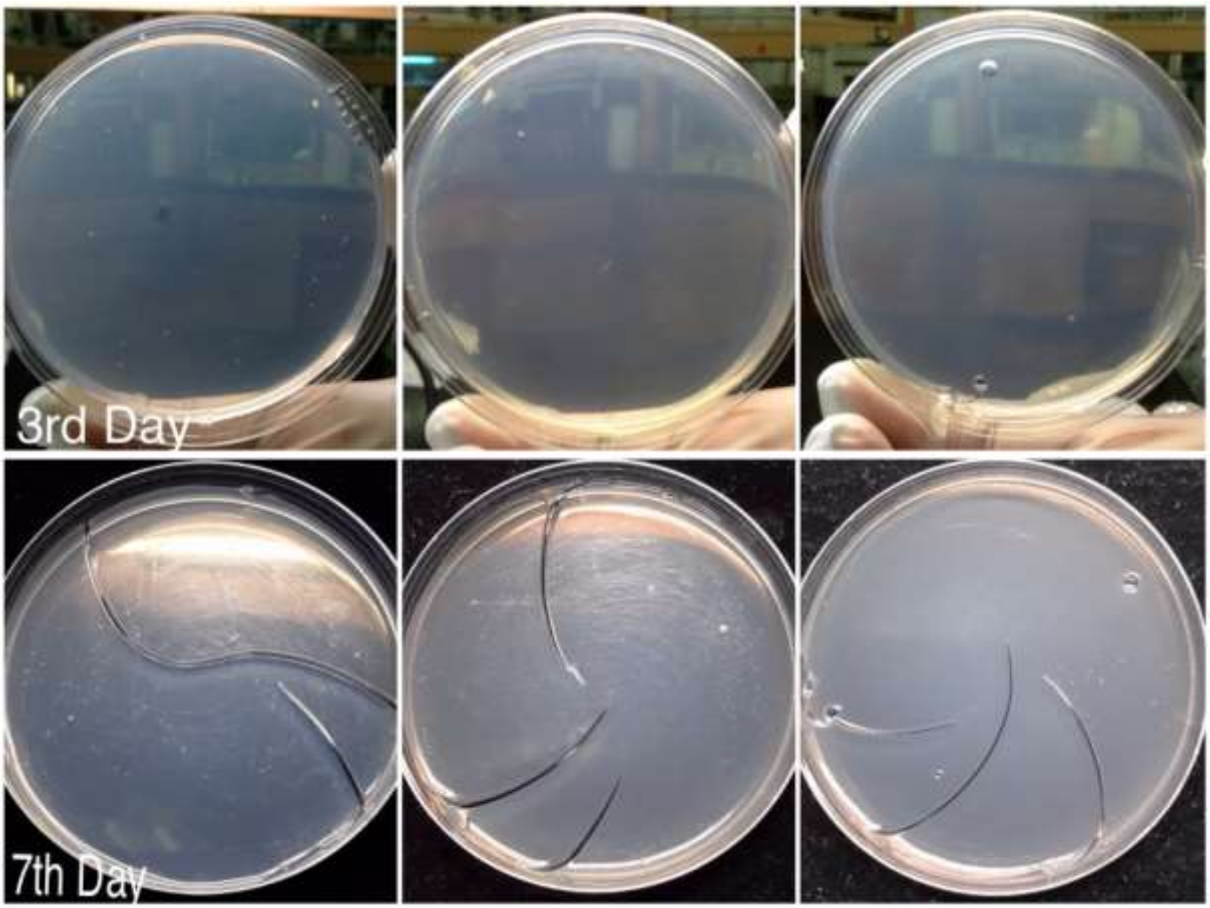


Figure 4.6. The growth of Achromobacter xylosoxidans F3B in the 3rd day and 7th day of Catechol exposure.

The *Achromobacter xylosoxidans* F3B also brought the same result as Burkholderia cenocepacia 869T2. The plates with 1.6 mM Catechol concentration had so few colonies of bacteria in the third day, and seemed to be no presence of bacteria anymore after a week of incubation.

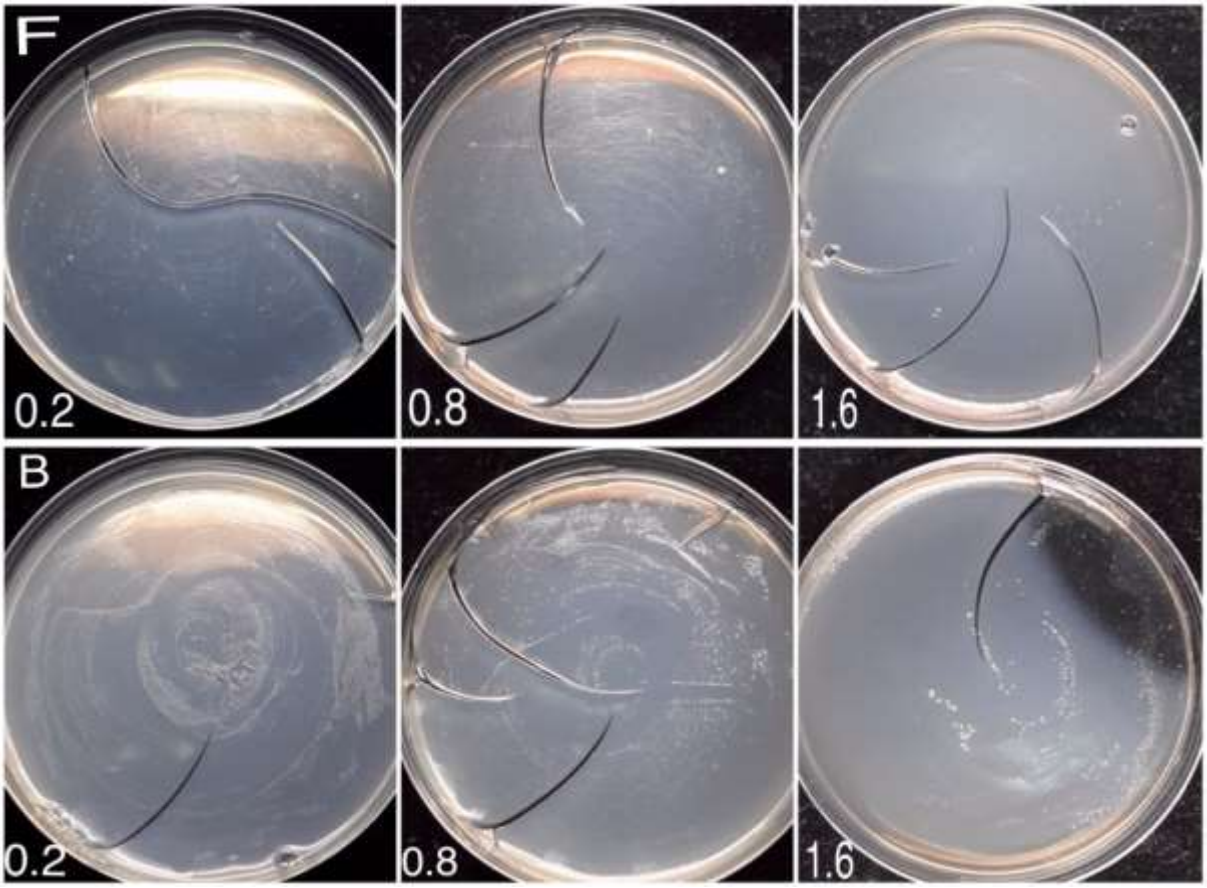


Figure 4.7. Comparison of the growth of Burkholderia cenocepacia 869T2 (B) and Achromobacter xylosoxidans F3B (F) after a week exposure to Catechol.

As shown in the figure 4.7, it is evident that *Burkholderia cenocepacia* 869T2 strain developed much better than *Achromobacter xylosoxidans* F3B train. There were few colonies of *A. xylosoxidans* F3B train in the plates, especially there were almost no colonies of *A. xylosoxidans* F3B in the plate which contains 1.6 mM Catechol. In one previous study demonstrated to have ability to degrade Catechol, however, the study results have indicated that *B. cenocepacia* 869T2 strain had more potential to tolerate and survive under the Catechol polluted environment than *A. xylosoxidans* F3B train.

4.3. Catechol Removal Test

After three days of incubation in the environment without carbon source and at temperature of 30 ^{o}C and 200 r.p.m, the Catechol concentration in the presences of

Burkholderia cenocepacia 869T2, *Achromobacter xylosoxidans* F3B and the control Catechol samples (no bacteria) had significant differences. The table 4.3 shows that the levels of Catechol of the control were fluctuated, and finally all of the three samples were declined at the third day. Because oxidization of Catechol caused the reduction of this chemical.

Table 4.3. The remaining concentration of Catechol for the control (without bacteria) in M9 medium after three days (Unit: mM).

The control (only Catechol)	1st Day	2nd Day	3rd Day
Sample 1	1.65	1.66	1.46
Sample 2	1.49	1.89	1.48
Sample 3	1.58	1.79	1.46

There are decreases in concentration of Catechol inside samples which includes B. cenocepacia 869T2 and A. xylosoxidans F3B, as shown on table 4.4 and 4.5. Particularly, it is notable that the remaining concentrations of Catechol in all samples of *B. cenocepacia* 869T2 were dramatically dropped to 0.0 mM, Catechol was degraded thoroughly after just 48 hours. In the environment without nutrient or no carbon source, *B. cenocepacia* 869T2 bacteria had effectively utilized Catechol for survival and growth.

Table 4.4. The remaining concentration of Catechol in the M9 medium in the presences of Burkholderia cenocepacia 869T2 after three days. (Unit: mM)

Burkholderia cenocepacia 869T2	1st Day	2nd Day	3rd Day
Sample 1	1.22	0	0
Sample 2	1.53	0	0
Sample 3	1.33	0	0

For *Achromobacter xylosoxidans* F3B, this endophyte also can eliminate Catechol, use and develop in the contaminated condition. However, the remaining level of Catechol is still a considerable number, around 1mM Catechol.

Table 4.5. The remaining concentration of Catechol in the M9 medium in the presences of Achromobacter xylosoxidans F3B after three days. (Unit: mM).

Achromobacter xylosoxidans F3B	1st Day	2nd Day	3rd Day
Sample 1	1.31	1.06	1.02
Sample 2	1.54	1.11	0.98
Sample 3	1.42	1.01	0.92

Table 4.6. The average concentration of catechol remaining in the M9 medium in the presences of Burkholderia cenocepacia 869T2, Achromobacter xylosoxidans F3B and the control for three day. (Unit: mM).

	1st Day	2nd Day	3rd Day
The control	1.57 ± 0.08	1.78 ± 0.11	1.47 ± 0.02
Burkholderia cenocepacia 869T2	1.36 ± 0.16	0 ± 0	0 ± 0
Achromobacter xylosoxidans F3B	1.42 ± 0.17	1.06 ± 0.05	0.98 ± 0.05

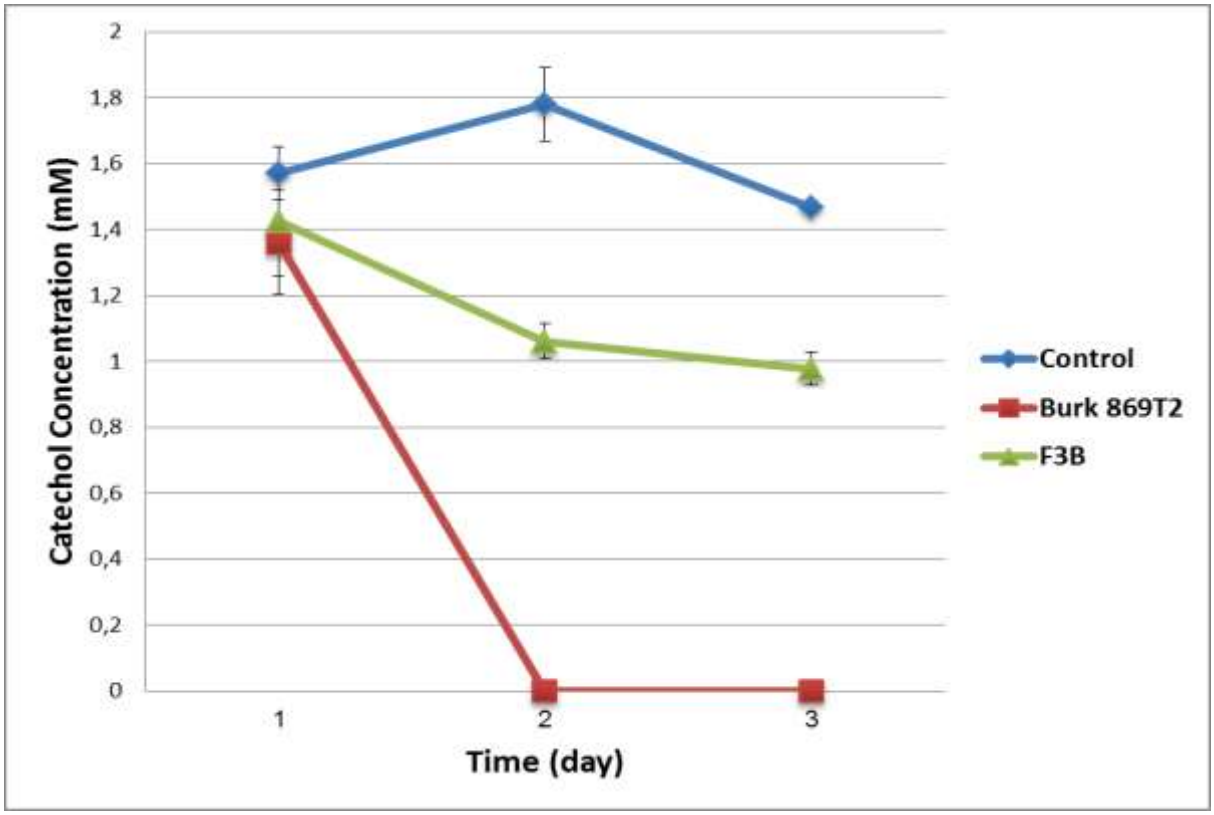


Figure 4.8. The average concentration of catechol remaining in the M9 medium in the presences of Burkholderia cenocepacia 869T2, Achromobacter xylosoxidans F3B and the control for three days.

The results indicated that *B. cenocepacia* 869T2 strain had a strong potential in removal of Catechol, and even were much better than the endophytic bacteria *A.*

xylosoxidans F3B that had introduced to have ability to degrade Catechol and other aromatic compounds in the previous researches. The endophytic *B. cenocepacia* 869T2 utilized Catechol as a carbon source for survival in that contaminated environment. Therefore, it is evident that *Burkholderia cenocepacia* 869T2 were extremely efficient in removing catechol pollutants.

PART V. DISCUSSION AND CONCLUSION

5.1. Discussion

Firstly, the effect of Catechol toxicity on plant growth. For the uninoculated control plants, in the environment without Catechol and bacteria, they had the best environment in compared with the others, thereby their root was and longest and they gained the largest amount of fresh weight. *Arabidopsis thaliana* plants could live in Catechol contaminated condition which was reportedly introduced in the previous study of Chieh-Chen Huang in National Chung Hsing University, Taiwan in 2012. For the plants inoculated with *Burkholderia cenocepacia* 869T2, the plants were colonized by bacteria and died. One explanation of this observation is the experimental method and chemical components used are not really valid, thus colonies of bacteria were too much and this colonization bacteria with the effects of Catechol pollutants caused death of plants. As expected, the *Burkholderia cenocepacia* 869T2 should potentially improve the growth of the inoculated *A. thaliana* plants to grow better under the aromatic compounds or catechol contamination as *Achromobacter xylosoxidans* F3B had been demonstrated to have ability to degrade Catechol and Phenol and help *Arabidopsis thaliana* plants against Catechol phytotoxicity. In the newest research, the *Burkholderia cenocepacia* 869T2 strain had been reported to be potential in decrease the incidence of Fusarium wilt disease in banana tree and promote banana tree growth. Nonetheless, in this study, *Burkholderia cenocepacia* 869T2 could not help plants tolerate Catechol stress. *Arabidopsis thaliana* is not a suitable host for *Burkholderia cenocepacia* 869T2, this endophytic bacteria strain needs specific hosts.

Secondly, the growth of bacteria in Catecol toxicity. In one previous study demonstrated to have ability to degrade Catechol, however, the study results have indicated that *B. cenocepacia* 869T2 strain had more potential to tolerate and survive under the Catechol polluted environment than *A. xylosoxidans* F3B train.

Finally, *B. cenocepacia* 869T2 strain had a strong potential in removal of Catechol, and even were much better than the endophytic bacteria *A. xylosoxidans* F3B that had introduced to have ability to degrade Catechol and other aromatic compounds in the previous researches. The endophytic *B. cenocepacia* 869T2 utilized Catechol as a carbon source for survival in that contaminated environment. In the mentioned-above results of the experiment in the bacteria growth in Catechol toxicity, *B. cenocepacia* 869T2 had proved growing stronger *A. xylosoxidans* F3B with in the M9 medium with three different concentration of Catechol. Therefore, it is evident that *Burkholderia cenocepacia* 869T2 were extremely efficient in removing catechol pollutants.

5.2. Conclusion

- For the uninoculated plants in the presence of catechol, the higher concentrations of Catechol were, the worse the growth of the uninoculated plants was. The functional endophytic bacteria *Burkholderia cenocepacia* 869T2 could not help *Arabidopsis thaliana* to tolerate Catechol phytotoxicity, and was unsuccessful in improvement of phytoremediation of Catechol contamination as expected.

- In the same treatment and without a carbon source, *B. cenocepacia* 869T2 strain developed better and had more potential to tolerate and survive than *A. xylosoxidans* F3B train under the Catechol polluted environment.

- *Burkholderia cenocepacia* 869T2 were discovered and proved that this endophyte can utilized Catechol compounds as carbon source, and has a strong ability to degradation of Catechol and even better than Achromobacter xylosoxidans F3B that had been demonstrated to have ability to degrade Catechol and Phenol and help *Arabidopsis thaliana* plants against Catechol phytotoxicity in other previous studies.

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