การบำบัดดินปนเปื้อนน้ำมันดีเซลโดยใช้ถั่วพร้า *Canavalia* sp. ที่ใส่เชื้อไรโซเบียมและอาบัสคูลาร์ไมคอร์ไรซา

นางสาวศิริยาภรณ์ จุฑาพฤทธิ์

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาพฤกษศาสตร์ ภาควิชาพฤกษศาสตร์
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2551
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

PHYTOREMEDIATION OF DIESEL CONTAMINATED SOIL BY JACK BEAN *Canavalia* sp. INOCULATED WITH RHIZOBIUM AND ARBUSCULAR MYCORRHIZA

Ms. Siriyaporn Juthapruth

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Botany

Department of Botany

Faculty of Science

Chulalongkorn University

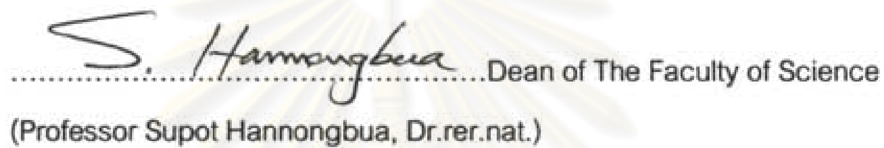
Academic Year 2008

Copyright of Chulalongkorn University

512054

Thesis Title	Phytoremediation of Diesel Contaminated Soil by Jack Bean *Canavalia* sp. Inoculated with Rhizobium and Arbuscular Mycorrhiza
By	Ms. Siriyaporn Juthapruth
Field of Study	Botany
Advisor	Teerada Wangsomboondee, Ph.D.

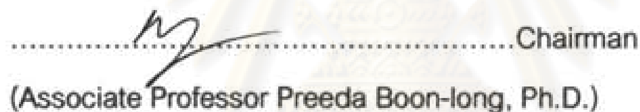
Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree



....Dean of The Faculty of Science

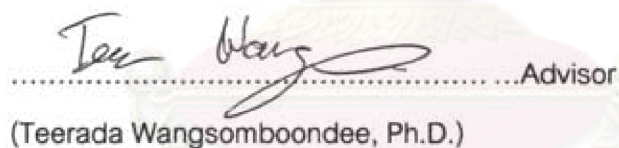
(Professor Supot Hannongbua, Dr.rer.nat.)

THESIS COMMITTEE



....Chairman

(Associate Professor Preeda Boon-long, Ph.D.)

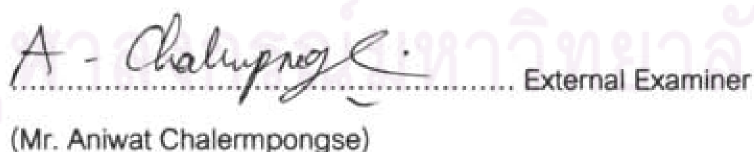


....Advisor

(Teerada Wangsomboondee, Ph.D.)

.... Examiner

(Assistant Professor Jittra Piapukiew, Ph.D.)



.... External Examiner

(Mr. Aniwat Chalermpongse)

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

ศิริยาภรณ์ จุฑาพฤทธิ์: การบำบัดดินปนเปื้อนน้ำมันดีเซลโดยใช้ถั่วพร้า *Canavalia* sp. ที่ใส่เชื้อไรโซเบียมและอาบัสคูลาร์ไมคอร์ไรซา. (PHYTOREMEDIATION OF DIESEL CONTAMINATED SOIL BY JACK BEAN *CANAVALIA* SP. INOCULATED WITH RHIZOBIUM AND ARBUSCULAR MYCORRHIZA) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: อ.ดร. ธีรดา หวังสมบูรณ์ดี, 101 หน้า

การบำบัดด้วยพืชเป็นวิธีหนึ่งที่ใช้ได้ผลดีในการบำบัดดินที่ปนเปื้อนเมื่อคำนึงถึงความเป็นไปได้ทั้งทางด้านเศรษฐกิจและสิ่งแวดล้อม แต่สิ่งสำคัญที่ต้องคำนึงถึงในการบำบัดด้วยพืชคืออัตราการเจริญของพืชในบริเวณที่มีการปนเปื้อนนั้นค่อนข้างต่ำ แต่ทั้งนี้พืชอาจอยู่ร่วมกันแบบพึ่งพาอาศัยกันกับจุลชีพ เพื่อให้พืชสามารถเจริญและบำบัดดินที่ปนเปื้อนได้ดียิ่งขึ้น ในการศึกษาวิจัยนี้ ได้ทำการศึกษาการบำบัดดินปนเปื้อนน้ำมันดีเซลโดยใช้ถั่วพร้า *Canavalia* sp. ที่ใส่เชื้อไรโซเบียมและอาบัสคูลาร์ไมคอร์ไรซา ทำได้โดยการคัดแยกอาบัสคูลาร์ไมคอร์ไรซาแล้วเพาะเลี้ยงในข้าวฟ่าง จากนั้นจึงกระตุ้นการเจริญของถั่วพร้าในดินที่ไม่ปนเปื้อนด้วยอาบัสคูลาร์ไมคอร์ไรซาปริมาณ 10 20 50 หรือ 100 สปอร์ หรือ ไรโซเบียม 10 กรัม เพียงอย่างเดียว หรือใส่ทั้งอาบัสคูลาร์ไมคอร์ไรซาและไรโซเบียม สามเดือนภายหลังการปลูกเชื้อปรากฏว่าการใส่อาบัสคูลาร์ไมคอร์ไรซาและไรโซเบียมในดินสามารถกระตุ้นการเจริญของถั่วพร้าและเพิ่มเปอร์เซ็นต์การติดเชื้ออาบัสคูลาร์ไมคอร์ไรซา จากการวิเคราะห์น้ำมันดีเซลที่ปนเปื้อนในดินหลังจากการบำบัดด้วยถั่วพร้า ที่ปลูกเชื้อด้วยอาบัสคูลาร์ไมคอร์ไรซา 100 สปอร์ ร่วมกับไรโซเบียม 10 กรัม พบว่าให้ผลในการลดปริมาณน้ำมันดีเซลที่ปนเปื้อนในดิน นอกจากนี้ยังตรวจพบไฮโดรคาร์บอนที่เป็นส่วนประกอบของน้ำมันดีเซลในต้นพืช แต่พบน้อยมากในราก แสดงถึงการเกิดการสะสมสารปนเปื้อนในพืช ซึ่งถือเป็นกลไกสำคัญในการกำจัดน้ำมันดีเซลที่ปนเปื้อนในดิน

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

ภาควิชา พฤกษศาสตร์
สาขาวิชา พฤกษศาสตร์
ปีการศึกษา 2551

ลายมือชื่อนิสิต.....น.ส. ศิริยาภรณ์ จุฑาพฤทธิ์.....
ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.........

4972615823 : MAJOR BOTANY

KEYWORDS: REMEDIATION / DIESEL CONTAMINATED SOIL / *CANAVALIA* SP.

GLOMUS MOSSEAE / RHIZOBIUM

SIRIYAPORN JUTHAPRUTH: PHYTOREMEDIATION OF DIESEL CONTAMINATED SOIL BY JACK BEAN *CANAVALIA* SP. INOCULATED WITH RHIZOBIUM AND ARBUSCULAR MYCORRHIZA. THESIS ADVISOR: TEERADA WANGSOMBOONDEE, Ph.D., 101 pp.

Phytoremediation is the outstanding way to remediate the diesel contaminated soil for both environmental and economic reasons but there is a major concern about the low bioavailability. Therefore the use of symbiosis of plants and microorganisms in order to stimulate the phytoremediation should be considered. In this study, phytoremediation of diesel contaminated soil by *Canavalia* sp. inoculated with rhizobium and arbuscular mycorrhiza (AM) was established. The AM fungi were isolated and cultivated in sorghums for inoculum production. *Canavalia* sp. was biostimulated in non contaminated soil with only AM at 10, 20, 50 or 100 spores or 10 g rhizobium or the mixture of both microorganisms. The results after three months inoculation showed that the presence of AM and rhizobium in soil significantly stimulated the growth of *Canavalia* sp. and increased %AM infection. Analysis of diesel contaminated soil after treating with *Canavaria* sp. co-inoculated with 100 spores AM with 10 g rhizobium resulted in the decrease of diesel contaminated in soil. In addition, hydrocarbon products of diesel oil were also detected in plant shoots but very little in roots. This phenomenon proved that the phytoaccumulation was occurred and played a role in diesel contaminated removal of soil.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Department: Botany
Field of study: Botany
Academic Year: 2008

Student's Signature. Siriyaporn Juthapruth
Advisor's Signature. Tee Nan

ACKNOWLEDGEMENTS

This thesis could not have successfully been possible and complete without the invaluable helps of the following individuals and organizations.

First of all, I would like to express my sincere gratitude to Dr. Teerada Wangsomboondee for her invaluable guidance, understanding, and constant encouragement throughout the course of this research. Her positive attitude significantly contributed to inspiring and maintaining my enthusiasm in the field.

I would like to express my sincere gratitude to Assoc. Prof. Dr. Preeda Boon-long, Asst. Prof. Dr. Jittra Piapukiew, and Mr. Aniwat Chalermpongse for their kind advice and for being on the thesis committee.

I am also grateful to the partial scholarship and partial funding of the thesis work provided by Graduate School, Chulalongkorn University. I wish to thank all the staffs at the Department of Botany and Department of Plant Science, Mahidol University for their kind assistance and cooperation on hydrocarbon analysis. Furthermore, I would also thank all of my friends for their unforgettable friendship.

Finally, my deepest appreciations are dedicated to Asst. Prof. Patchra Limpanavech and my beloved family whose endless support and understanding play the greatest role in my success.

CONTENTS

Page

ABSTRACT (THAI) iv

ABSTRACT (ENGLISH) v

ACKNOWLEDGEMENTS vi

CONTENTS vii

LIST OF TABLES x

LIST OF FIGURES xi

CHAPTER I INTRODUCTION 1

CHAPTER II BACKGROUND AND LITERATURE SURVEY 4

2.1 Diesel oil contaminated soil 4

2.2 Phytoremediation 6

2.3 Enhancement of phytoremediation 10

2.4 Mechanisms of phytoremediation to eliminate Petroleum hydrocarbons 11

2.4.1 Degradation 11

2.4.1.1 The rhizosphere effect 11

2.4.1.2 Role of plants in degradation 13

2.4.1.3 Role of microorganisms in degradation 17

2.4.2 Containment 20

2.4.3 Transfer of petroleum hydrocarbons to the atmosphere 21

2.5 Arbuscular mycorrhiza 21

2.5.1 Characteristics of arbuscular mycorrhiza 21

2.5.2 Classification of arbuscular mycorrhizal fungi 22

2.5.3 Role of arbuscular mycorrhizal fungi and minerals uptake 24

2.5.4 AM formation 25

2.5.5 AM and plant symbiosis 26

2.5.5.1 Soil hyphae 26

Page

2.5.5.2 Root contact and penetration ... 27

2.5.5.3 Arbuscules ... 27

2.5.5.4 Vesicles ... 27

2.5.5.5 Spores ... 28

2.6 Rhizobium-induced nodule formation ... 28

2.6.1 Rhizobium ... 28

2.6.2 Nod factors ... 29

2.6.3 Nodulation process ... 30

2.6.4 Host specificity ... 33

2.7 Interaction of soil microorganisms ... 35

2.7.1 AM-rhizobacteria interaction ... 35

2.7.2 Plant growth promoting rhizobacteria ... 37

2.7.3 AM-rhizobial interaction ... 38

2.7.4 AM and rhizobium induced phytoremediation of contaminated ... 38

2.8 Characterizations ... 39

2.8.1 Gas chromatography ... 39

2.8.2 Gas chromatography-mass spectrometry ... 40

CHAPTER III MATERIALS AND METHODS ... 42

3.1 Arbuscular mycorrhiza isolation and inoculums preparation ... 42

3.1.1 Wet sieving and decanting technique ... 43

3.1.2 Sucrose centrifugation ... 43

3.1.3 Inoculum preparation ... 44

3.1.4 Isolation and propagating of AM ... 44

3.1.5 Identification of selected AM fungi ... 45

3.2 Rhizobium preparation ... 46

3.3 Biostimulation by AM and rhizobium ... 46

3.4 Phytoremediation ... 48

Page

3.5 Analytical procedures 49

CHAPTER IV RESULTS AND DISCUSSIONS 51

RESULTS 51

4.1 AM fungal characteristic 51

4.2 Biostimulation 54

4.3 Effect of microorganisms on enhancing plant growth on contaminated soil 56

4.4 Phytoremediation 59

4.4.1 Analysis of pure diesel oil 59

4.4.2 Analysis of diesel oil extracted from soil 62

4.4.3 Analysis of diesel oil extracted from shoot and root 64

DISCUSSIONS 66

CHAPTER V CONCLUSIONS 71

REFERENCES 73

APPENDICES 89

CURRICULUM VITAE 101

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

LIST OF TABLES

Table		Page
2.1	Potential use of plant in phytoremediation of contaminated soil	8
2.2	Classification scheme for AM taxa	23
3.1	Plant species and sampling site for AM collection	43
3.2	Detailed amendment of each biostimulation experiment	47
3.3	Detailed amendment of each phytoremediation experiment	49
4.1	The number of spore production and %AM infection of each plant species	52
4.2	Plant growth parameters and biomass of Jack bean after biostimulation	52
4.3	Comparing of plant growth parameters, biomass, and %AM infection of Jack bean grown on normal soil and diesel contaminated soil	57
4.4	Major constituents of pure diesel oil	61
4.5	Major constituents of diesel oil extracted from soil	63
4.6	Major constituents of diesel oil extracted from shoot	65

LIST OF FIGURES

Figure | Page

2.1 Nod factors produced by *Sinorhizobium meliloti* and *Rhizobium leguminosarum* biovar *viciae* 30

2.2 Gas chromatograph compartments 39

3.1 A three-month-old pot culture of *Sorghum bicolor* 44

3.2 Agilent Technologies 6890 gas chromatograph 50

4.1 *Glomus mosseae* spores 52

4.2 Isolated spore from *Acacia mangium* Identified into genus *Glomus mosseae*, L: spore wall layer 53

4.3 Appearances of plant growth amended with AM and/or rhizobium 54

4.4 Appearances of plant growth on normal soil and contaminated soil 58

4.5 Gas chromatograms of pure diesel oil 59

CHAPTER I

INTRODUCTION

Petroleum hydrocarbons are naturally occurring chemicals used by humans for a variety of activities, including the fueling of vehicles and heating of homes. Diesel oil, natural gas, crude oil, tars and asphalts are types of petroleum hydrocarbons ultimately composed of various proportions of alkanes (e.g., methane, ethane, propane), aromatics (e.g. benzene, toluene, ethylbenzene, and xylene, collectively known as BTEX), and polycyclic aromatic hydrocarbons (PAHs; e.g., naphthalene, phenanthrene, anthracene, benzo[a]pyrene). During the past century, industrialization has resulted in an ever-increasing reliance on petrochemicals. This, in turn, has resulted in the contamination of a significant number of sites with petroleum and petroleum-byproducts.

Diesel oil pollutants in soil generally have strong negative effects on the plant and animal community both direct contact toxicity and inderect effects by interactions of the diesel oil pollutants with the abiotic and microbial components of soil (Bossert et al. 1984). It has been recognized that certain hydrocarbon constituents commonly found in diesel oil have some carcinogenic and mutagenic potential which associated with polycyclic aromatic hydrocarbon (PAH) fraction (Wang and Bartha, 1994).

Recently, heightened environmental awareness and government regulation effort to cleanup the contaminated sites represent both a commitment to responsible stewardship of our limited natural resources and good business. Today, environmental managers can choose from a variety of approaches to remediate diesel contaminated soil. These approaches range from intensive engineering techniques to natural attenuation, a "hands-off" approach relying entirely on natural processes to remediate sites with no human intervention.

Phytoremediation is a natural attenuation of contaminated soil by the uses of certain plants for a specific site. In essence, phytoremediation employs human initiative to enhance the natural attenuation of contaminated sites and, as such, is a process that is intermediate between engineering and natural attenuation. Because phytoremediation depends on natural, synergistic relationships among plants, microorganisms and the

environment, it does not require intensive engineering techniques or excavation. Human intervention may be required to establish an appropriate plant-microbe community at the site or apply agronomic techniques (such as tillage and fertilizer application) to enhance natural degradation or containment processes. Phytoremediation has been used effectively to remediate inorganic and organic contaminants in soil. The application and effectiveness of phytoremediation depend on the nature of the compounds to be remediated.

Certain plants have been found to decompose a limited range of hydrocarbon compounds in soil. Planting alfalfa and horseradish in soil were reported to reduce concentration of kerosene-based jet fuel by 57 – 90 % in 5 months (Karthikeyan et al. 1999). Various varieties of alfalfa (*Medico sativa* L.) were also capable of reducing crude oil contamination in the rhizosphere by 33 56 % (Wiltse et. al, 1998) and reduced 80 % diesel fuel in soil after 8 weeks of treatment (Komisar and Park, 1997). Broad bean (*Vicia faba*) has been reported (Radwan et al. 1998) to removed 46 % of crude oil within 12 weeks compared to 33 % with no plants.

Furthermore, introduction of fertilizers into oil-contaminated, nonvegetated soil has been shown to enhance the degradation of hydrocarbons via biostimulation (Venosa et al. 1996). The main potential obstacles that need to be overcome to improve phytoremediation systems are low bioavailability of the pollutants due to adsorption to soil particles (Cunningham et al. 1996) and lack of adequate microbial activities (Armishaw et al. 1991). Plants may support a microflora in the rhizosphere with much greater adaptability for growth on different carbon sources including pollutants than non-rhizosphere microflora (Siciliano et al. 1998). In addition, certain legumes support symbiotic rhizobial species that fix atmospheric nitrogen and improve the nutrient status of a contaminated soil and enhance the degradation of pesticides (Anderson et al. 1994), or polyaromatic hydrocarbons (Reilly et al. 1996; and Joner et al. 2001). Furthermore, establishing symbiosis of plants and arbuscular mycorrhiza resulted in reciprocal transfer of phosphorus from the fungus to the plant in exchange for carbons from the plant to the fungus (Ezawa et al. 2002). Dual inoculation of legumes with rhizobia and arbuscular mycorrhiza can increase plant growth (Mosse et al. 1976; Redente and

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Reeves, 1981; Abd-Alla, 2000). However, no studies have examined the effect of both rhizobial and mycorrizal inoculum with plant during remediation of diesel oil.

In the present study, the effects of rhizobium and mycorrhiza in a legume plant were determined to enhance phytoremediation of diesel oil contaminated soil.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER II

BACKGROUND AND LITERATURE REVIEWS

2.1 Diesel oil contaminated soil

Diesel is a specific fractional distillate of petrolium fuel oil used in diesel engines. Over the past century, mining, manufacturing and urban activities have involved in the use of diesel and all contributed to extensive soil contamination. Deisel oil pollutants exposures are most frequently the result of accidents such as leak containers, drilling, and transportation that once introduced into the soil environment, they undergo a variety of physical, chemical, and metabolic transformations. Some of these transformations may detoxify and eventually eliminate the pollutants. Other transformations will increase the toxicity of the pollutants as such as photochemical and microbial oxidations, often through free radical mechanisms, produce aliphatic and aromatic ketones, aldehydes, carboxylic acids, esters, epoxides, sulfoxides, sulfones, phenols, and alcohols (Wang and Bartha, 1994).

It has been recognized for many years that certain hydrocarbon constituents commonly found in diesel oil have some carcinogenic and mutagenic potential. This mutagenic and carcinogenic potential is associated with the polycyclic aromatic hydrocarbon (PAH) fraction. These PAHs have to be enzymatically metabolized to chemically reactive electrophiles to initiate the carcinogenic process by covalent interaction with DNA (Wang and Bartha, 1994).

Diesel oil pollutants in soil generally have strong negative effects on the plant community both direct contact toxicity and inderect effects by interactions of the diesel oil pollutants with the abiotic and microbial components of soil. Contact toxicity occurs primatily by the solvent effect of low-boiling hydrocarbons on the lipid membrane structures of the cells. Diesel oil also affects the physical structure of soil, decreasing its capacity to store moisture and air. Indirect effects of diesel oil pollutants in soil include

oxygen deprivation of plant roots, due to the exhaustion of soil oxygen by hydrocarbon-degrading microorganisms and may bring about the microbial production of phytotoxic compounds such as H_2S. Hydrocarbon-degrading microorganisms also compete with plants for mineral nutrients. The metabolic intermediates of hydrocarbon-degrading microorganisms may temporarily increase the toxicity of hydrocarbon pollutants including fatty acids, phenolic and terpenoid compounds, which all possess phytotoxic properties (Wang and Bartha, 1994).

Various physical, chemical and biological processes are already being used to remediate contaminated soil. These processes either decontaminate or stabilize the pollutant within it. Decontamination reduces the amount of pollutants within the soil by removing them, while stabilization does not reduce the quantity of pollutant at a site, but makes use of soil amendments to alter the soil chemistry and absorb the pollutant into the matrix so as to reduce or eliminate environmental risks (Wang and Bartha, 1994).

The choice of remediation strategy depends on the nature of the contaminants. Metal contaminants are difficult to remediate. Soils that are contaminated with metals are usually excavated and landfilled, although some sites are now treated by acid leaching, physical separation of the contaminant, or electrochemical processes. Soils contaminated with organics are treated by vapor stripping or thermal desorption (for volatiles and semi-volatiles), soil washing (for leachable materials), incineration (for all organics not otherwise treatable) and some landfilling. Certain organic contaminants, primarily petroleum hydrocarbons, are amenable to microbiological treatment, although the volume of material currently treated in this manner is relatively small. Biological soil-treatment systems include landfarming of some petroleum hydrocarbons, and *Ex Situ* techniques such as composting, bio-piles and slurry reactors. The costs associated with soil remediation are highly variable and depend on the contaminant, soil properties, site conditions and the volume of material to be remediated. Techniques that remediate a soil *In Situ* are generally less expensive than those that require excavation (Wang and Bartha, 1994).

2.2 Phytoremediation

Phytoremediation is the use of certain plants to clean up soil, sediment, and water contaminated with metals and/or organic contaminants such as hydrocarbon oil, solvents, and polyaromatic hydrocarbons (PAHs) by selecting the proper plant and conditions for a specific site. It is a mechanism that can reduce remediation costs, restore habitat, and clean up contamination in a place rather than transporting the problem to another site. Phytoremediation can be used to clean up contamination in several ways comprising of;

Phytovolatilization: Plants take up water and organic contaminants through the roots, transport them to the leaves, and release the contaminants as a reduced of detoxified vapor into the atmosphere (Tollsten et al. 1997).

Microorganism stimulation: Plants excrete and provide enzymes and organic substances from their roots that stimulate growth of microorganisms such as fungi and bacteria. The microorganisms in the rhizosphere then metabolize the organic contaminants (Jones et al. 2004).

Phytostabilization: Plants prevent contaminants from migrating by reducing run off, surface erosion, and ground-water flow rates. Hydraulic pumping can occur when tree roots reach ground water, take up large amounts of water, control the hydraulic gradient, and prevent lateral migration of contaminants within a ground water zone (Berti and Cuningham 2000).

Phytoaccumulation/extraction: Plant roots can remove metals from contaminated sites and transport them to leaves and stems for harvesting and disposal or metal recovery through smelting processes (Salt et al. 1998).

Phytodegradation: Organic contaminants are absorbed inside the plant and metabolized to non-toxic molecules by natural chemical processes within the plant (Salt et al. 1998).

The advantages of using phytoremediation are that it is stable to work on a variety of organic and inorganic compounds, either *In Situ/Ex Situ*, easy to implement and maintain low-cost compared to other treatment methods, and also environmental

friendly. In contrast, the disadvantage aspects that restrain the use of phytoremediation consist of restricted to sites with shallow contamination within rooting zone, may take several years to remediate and depend on climatic conditions, harvested biomass from phytoextraction may be classified as a hazardous waste and consumption of contaminated plant tissue is also a concern. Thus more fieldwork and analysis are necessary to understand the possible effects of phytoremediation.

Various plants have been identified for their potential to facilitate the phytoremediation of sites contaminated with petroleum hydrocarbons. In the majority of studies, grasses and legumes have been singled out for their potential in this regard (Aprill and Sims, 1990; Qiu et al. 1997; Gunther et al. 1996; Reilley et al. 1996). Prairie grasses are thought to make superior vehicles for phytoremediation because they have extensive, fibrous root systems. Grass root systems have the maximum root surface area of any plant type and may penetrate the soil to a depth of up to 3 m (Aprill and Sims, 1990). They also exhibit an inherent genetic diversity, which may give them a competitive advantage in becoming established under unfavorable soil conditions. Legumes are thought to have an advantage over non-leguminous plants in phytoremediation because of their ability to fix nitrogen; i.e., legumes do not have to compete with microorganisms and other plants for limited supplies of available soil nitrogen at oil-contaminated sites (Gudin and Syratt, 1975). Other plants identified for their potential use in phytoremediation are showed in Table 2.1.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Table 2.1 A brief summary of plants used for phytoremediation of petroleum hydrocarbons.

Plant	Type of contamination	Reference
- Alfalfa (*Medico sativa* L.)	Crude oil	Wiltse et al. 1998 Komisar and Park, 1997 Radwan et al. 2000
- Alfalfa (*Medicago sativa* L.) - Horseradish (*Armoracia rusticana*)	Kerosene-based jet fuel	Karthikeyan et al. 1999
- Alfalfa (*Medicago sativa* L.) - Tall fescue (*Festuca arundinacea* Schreb.) - Sudangrass (*Sorghum vulgare* L.) - Switchgrass (*Panicum virgatum*)	- Anthracene - Pyrene	Reilley et al. 1996
- Alfalfa (*Medicago sativa* L.) - Switchgrass (*Panicum virgatum*) - Little bluestem (*Schizachyrium scoparius*)	PAHs	Pradhan et al. 1998
- Alfalfa (*Medicago sativa* L.) - Sorghum (*Sorghum bicolor*) - Bermuda grass (*Cynodon dactylon* L.)	[^{14}C] Phenanthrene	Schwab et al. 1995
- Alfalfa (*Medicago sativa* L.) - White clover(*Trifolium repens*) - Birdsfoot trefoil (*Lotus corniculatus*) - Black medick (*Medicago lupulina*)	Oil	Gudin and Syratt, 1975
- Maize (*Zea mays* L.)	- Saturated hydrocarbons - Aromatic hydrocarbons	Chaineau et al. 2000
- Arctared red fescue (*Festuca rubra* var. Arctared) - Annual ryegrass (*Lolium multiflorum*)	Crude oil	Reynolds and Wolf , 1999

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Table 2.1 (Con't) A brief summary of plants used for phytoremediation of petroleum hydrocarbons.

Plant	Type of contamination	Reference
Ryegrass (*Lolium perenne* L.)	- *n*-alkanes (C_{10} to C_{24}) - Phenanthrene - Anthracene - Fluoranthene - Pyrene	Gunther et al. 1996
- Big bluestem (*Andropogon gerardi*) - Little bluestem (*Schizachyrium scoparius*) - Indiangrass (*Sorghastrum nutans*) - Switchgrass (*Panicum virgatum*) - Canada wild-rye (*Elymus canadensis*) - Sideoatsgrama (*Bouteloua curtipendula*) - Blue grama (*Bouteloua gracilis*) - Wwestern wheatgrass (*Agropyron smithii*)	PAHs - Benzo[a]pyrene, - Benzo[a]anthracene, - Dibenzo[a,h]anthracene, - Chrysene	Aprill and Sims, 1990
- Prairie buffalograss (*Buchloe dactyloides* var. Prairie) - Common buffalograss (*Buchloe dactyloides*) - Meyer zoysiagrass (*Zoysia japonica* var. Meyer) - Verde kleingrass (*Panicum coloratum* var. Verde)	Low molecular weight PAHs - Naphthalene - Fluorene - Phenanthrene High molecular weight PAHs - Pyrene - Benzo[a]anthracene - Benzo[a]pyrene	Qiu et al. 1997

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

2.3 Enhancement of phytoremediation

The main potential obstacles that need to be overcome to improve phytoremediation systems are low bioavailability of the pollutants due to adsorption to soil particles (Johnson et al. 2002), and lack of adequate microbial activity (Armishaw et al. 1991). Introduction of fertilisers into oil-contaminated, nonvegetated-soil has been shown to enhance the degradation of hydrocarbons via biostimulation (Venosa et al. 1996). Plants may enhance microbial degradation by providing specific microenvironments for pollutant degradation or symbiotic microorganisms. Plants may support a microflora in the rhizosphere with much greater adaptability for growth on different carbon sources including pollutants than non-rhizosphere microflora (Siciliano et al. 1998). Many studies have shown that microbial counts increase in soil contaminated with hydrocarbons when alfalfa was introduced into the area (Komisar and Park, 1997).

Certain legumes support symbiotic rhizobial species that fix atmospheric nitrogen and improve the nutrient status of a contaminated soil. There is evidence that microbial activity in the rhizosphere may be used to enhance the degradation of pesticides (Anderson et al. 1994) and persistent industrial chemicals such as polyaromatic hydrocarbons (Reilly et al. 1996; Joner et al. 2001). Rhizobium likely to play an important role due to their ability to alleviate nutrient limitations and thus increase plant and root growth. It is known that rhizobium can increase exudation from host roots which may support the growth of microorganisms or influence pollutant availability (Phillips and Streit, 1996). Furthermore, the catabolic diversity of rhizobia has been known shown to have the ability to degrade haloaromatics and other complex compounds such as polychlorinated biphenyls (Damaj and Ahmad, 1996).

Plants can establish symbiosis with arbuscular mycorrhiza, which may result in reciprocal transfer of phosphorus from the fungus to the plant in exchange for carbon from the plant to the fungus (Ezawa et al. 2002). Arbuscular mycorrhiza are widespread occurrence and may represent the natural status of most tropical plant species (Siqueira et al. 1998). A recent study on the role of mycorrhiza during the remediation of PAHs in a

mixed of clover and ryegrass showed the ability to enhance losses of chrysene and dibenzo(a,h)anthracene in a planted soil containing a mycorrhizal inoculum (Joner et al. 2001). Dual inoculation of legumes with rhizobia and arbuscular mycorrhiza can increase plant growth (Mosse et al. 1976, Redente and Reeves, 1981, Abd-Alla et al. 2000).

2.4 Mechanisms of phytoremediation to eliminate Petroleum hydrocarbons

There are three primary mechanisms by which plants and microorganisms remediate petroleum-contaminated soil. These mechanisms include degradation and containment, as well as transfer of the hydrocarbons from the soil to the atmosphere (Cunningham et al. 1996; Siciliano and Germida, 1998; Sims and Overcash, 1983). The following section provides a detailed discussion of these mechanisms.

2.4.1 Degradation

Plants and microorganisms are involved, both directly and indirectly, in the degradation of petroleum hydrocarbons into products (e.g., alcohols, acids, carbon dioxide, and water) that are generally less toxic and less persistent in the environment than the parent compounds (Eweis et al. 1998). Though plants and microorganisms can degrade petroleum hydrocarbons independently of one another, the literature suggests that it is the interaction between plants and microorganisms which is the primary mechanism responsible for petrochemical degradation in phytoremediation efforts.

2.4.1.1 The rhizosphere effect

The rhizosphere is the region of soil closest to the roots of plants and is, therefore, under the direct influence of the root system. Plants provide root exudates of carbon, energy, nutrients, enzymes and sometimes oxygen to microbial populations in

the rhizosphere (Cunningham et al. 1996; Campbell, 1985; Vance, 1996). Root exudates of sugars, alcohols, and acids can provide sufficient carbon and energy to support large numbers of microbes (e.g., approximately 10^8 – 10^9 vegetative microbes per gram of soil in the rhizosphere; Erickson et al. 1995). Due to these exudates, microbial populations and activities are 5 to 100 times greater in the rhizosphere than in bulk soil (Atlas and Bartha, 1998; Gunther et al. 1996; Anderson et al. 1993; Paul and Clark, 1989). This plant induced enhancement of the microbial population is referred to as the rhizosphere effect (Atlas and Bartha, 1998) and is believed to result in enhanced degradation of organic contaminants in the rhizosphere.

Several studies serve as examples of the rhizosphere effect in the phytoremediation of petroleum hydrocarbons. Gunther et al. (1996) found higher microbial numbers and activities coupled with increased degradation in hydrocarbon contaminated soil planted with ryegrass compared to unplanted soil. The authors suggested that plant roots stimulated the microbes, which enhanced the degradation of the hydrocarbon mixture. Jordahl et al. (1997) reported that populations of microorganisms capable of degrading benzene, toluene, and xylene were five times more abundant in the rhizosphere of poplar trees (*Populus deltoides* x *nigra* DN-34, Imperial Carolina) than in bulk soil. Likewise, Radwan et al. (1998) identified that the roots of several plants from the Kuwaiti desert (*Senecio glaucus, Cyperus conglomeratus, Launaea mucronata, Picris babylonica* and *Salsola imbricata*) and crop plants (*Vicia faba* and *Lupinus albus*) were densely associated with hydrocarbon-utilizing bacteria (*Cellulomonas flavigena, Rhodococcus erythropolis* and *Arthrobacter* species). The rhizosphere soils of all plants contained greater numbers of these hydrocarbon-utilizing bacteria than bulk soils and this rhizosphere effect was more pronounced for plants growing in oil-contaminated soil compared to clean soil. As a result, the authors suggested that phytoremediation may be a feasible approach for cleaning oil-polluted soils.

2.4.1.2 Role of plants in degradation

Evidence regarding the direct degradation of petroleum hydrocarbons by plants is somewhat dated and limited in quantity. Durmishidze (1977) summarized various studies, primarily from the Union of Soviet Socialist Republic (USSR), on degradation pathways of hydrocarbons in plants. Corn seedlings, tea, and poplar shoots were reported to metabolize methane into various acids. The assimilation of radiolabelled methane, ethane, propane, butane, and pentane was recorded for bean and corn seedlings, as well as tea, grape, walnut and quince. The ability to assimilate *n*-alkanes and liberate $^{14}CO_2$ was identified in leaves and roots of both whole and cut plants. The general pathway of conversion for alkanes in plants was generalized as:

n-alkane > primary alcohols > fatty acids > acetyl-CoA > various compounds

Durmishidze (1977) also reported that benzene, toluene, and xylene were metabolized by cereal grasses in only two to three days; by the green mass of corn in four to five days; and by root crops in five to six days. Phenol was reported as the primary conversion product of benzene in plant tissues, with the subsequent production of various acids. The primary cleavage products of toluene were given as glycol, as well as glyoxalic, fumaric, succinic, and malic acid. Benzo[*a*]pyrene was reportedly metabolized by 14-day-old corn and bean plants, alfalfa, ryegrass, chick pea, cucumbers, squash, orchard grass, and vetch, with the amount of degradation ranging from 2 to 18% of the benzo[a]pyrene taken up by the plant and varying with plant type. The results of several other more recent studies also indicate that PAHs can be degraded directly by plants. Edwards (1988) documented the metabolism of [^{14}C]anthracene and [^{14}C]benz[*a*]anthracene in bush bean grown in a nutrient solution containing the two PAHs. Within the plant, parent compounds were transformed into both polar and non-polar metabolites. Interestingly, substantial quantities of the polar metabolites moved into the nutrient solution as root exudates. By maintaining sterile conditions the author ruled out microbial transformation as the pathway for the

production of these metabolites. Edwards et al. (1982) also reported that soybean was capable of degrading [^{14}C]anthracene. Evidence of the degradation was given by measuring the $^{14}CO_2$ given off from plants placed in [^{14}C]anthracene contaminated soil. It was also measured by analyzing extracts from plants that had their roots placed in a solution containing [^{14}C]anthracene.

There is a considerable body of information available regarding the indirect roles played by plants in the degradation of petroleum hydrocarbons. These include: (i) the supply of root exudates that cause the rhizosphere effect and enhance cometabolic degradation, (ii) the release of root-associated enzymes capable of transforming organic pollutants, and (iii) the physical and chemical effects of plants and their root systems on soil conditions (Gunther et al. 1996).

2.4.1.2.1 Root exudates

As described above, root exudates are the link between plants and microbes that leads to the rhizosphere effect. The type and amount of root exudate are depend on plant species and the stage of plant development. For example, Hegde and Fletcher (1996) found that the release of total phenolics by the roots of red mulberry (*Morus rubra* L.) increased continuously over the life of the plant with a massive release at the end of the season accompanying leaf senescence. The type of root exudate is also likely to be site and time specific (Siciliano et al. 1998). Site and time factors include variables such as soil type, nutrient levels, pH, water availability, temperature, oxygen status, light intensity, and atmospheric carbon dioxide concentration.

The type of root exudate can influence the type of interaction between plants and soil microorganisms. For instance, interactions can be "specific" or "non-specific" depending on the exudate. Specific interactions occur when the plant exudes a specific compound in response to the presence of a contaminant (Siciliano et al. 1998). Non-specific interactions occur when typical or normal plant exudates are chemically similar to the organic contaminant, resulting in increased microbial activity and increased degradation of the contaminants (Siciliano et al. 1998). For example, the

roots of red mulberry typically exude rhizosphere phenolics that help create a suitable environment for the biodegradation of PCBs, and perhaps PAHs, by selectively promoting the growth of certain microbes, such as the PCB-degrading bacteria *Alcaligenes eutrophus* H850, *Corynebacterium* sp. MB1, and *Pseudomonas putida* LB400 (Hegde and Fletcher, 1996; Donnelly et al. 1994). Results of experiments using rhizosphere soil collected from alfalfa roots and non-rhizosphere (bulk) soil suggest that the continued presence of plant roots and their exudates may be required for degradation of PAHs in soil (Wetzel et al. 1997). Conversely, reduced mineralization of naphthalene was found in soils planted with bell rhodesgrass (*Chloris gayana*) versus unplanted soils, possibly because root exudates may have placed naphthalene-degrading microorganisms at a competitive disadvantage (Watkins et al. 1994).

2.4.1.2.2 Cometabolism

Cometabolism is the process by which a compound that cannot support microbial growth on its own can be modified or degraded when another growth-supporting substrate is present (Cunningham and Berti, 1993). Organic molecules, including plant exudates, can provide energy to support populations of microbes that co-metabolize petroleum hydrocarbons. For example, Ferro et al. (1997) hypothesized that plant exudates may have served as cometabolites during the biodegradation of [^{14}C]pyrene in the rhizosphere of crested wheatgrass. Petroleum hydrocarbons can also serve as cometabolites, particularly for the larger, more persistent (i.e., recalcitrant) hydrocarbons such as PAHs with four or more benzene rings. Indeed, the presence of oil and grease co-substrates significantly enhanced the degradation of fluoranthene, pyrene, indo[1,2,3-*c,d*]pyrene, benz[*a*]anthracene, benzo[*k*]fluoranthene, chrysene, and benzo[*g,h,i*]perylene, all of which have four or five benzene rings (Keck et al. 1989). Benzo[a]pyrene is another large (five-ring) PAH that is typically recalcitrant in soil, yet it was almost completely degraded (95% degradation) by soil microbes when suitable cosubstrates were present in a crude oil mixture (Kanaly et al. 1997). The recalcitrant nature of PAHs with four or more benzene rings is thought to be due to the inability of

microorganisms to use these compounds directly for energy and growth, which emphasizes the importance of cometabolism in their degradation (Kanaly et al. 1997; Keck et al. 1989; Sims and Overcash, 1983).

2.4.1.2.3 Plant enzymes involved

Another indirect role that plants play in the degradation of petroleum hydrocarbons involves the release of enzymes from roots. These enzymes are capable of transforming organic contaminants by catalyzing chemical reactions in soil. Schnoor et al. (1995) identified plant enzymes as the causative agents in the transformation of contaminants mixed with sediment and soil. Isolated enzyme systems included dehalogenase, nitroreductase, peroxidase, laccase, and nitrilase. These findings suggest that plant enzymes may have significant spatial effects extending beyond the plant itself and temporal effects continuing after the plant has died (Cunningham et al. 1996).

2.4.1.2.4 Effect of plants on physical/chemical soil condition

Plants and their roots can indirectly influence degradation by altering the physical and chemical condition of the soil. Soil exploration by roots helps bring plants, microbes, nutrients and contaminants into contact with each other. Plants also provide organic matter to the soil, either after they die or as living plants through the loss of root cap cells and the excretion of mucigel, a gelatinous substance that is a lubricant for root penetration through the soil (Cunningham et al. 1996). Organic matter can reduce the bioavailability (i.e., the extent to which a contaminant is available to interact with living organisms) of some petroleum hydrocarbons, particularly those that are lipophilic (soluble in lipids) and bind to organic matter.

2.4.1.3 Role of microorganisms in degradation

Currently, microorganisms are used to destroy or immobilize organic contaminants in the absence of plants in a process referred to as bioremediation. These issues include the types of microorganisms involved in phytoremediation, reasons for microbial degradation, differences in degradation by various microorganisms, characteristics of microbial communities involved in degradation, and the role microorganisms play in reducing phytotoxicity to plants.

2.4.1.3.1 Types of microorganisms

A variety of microorganisms are reportedly involved in the degradation of petroleum hydrocarbons. In general, the bacteria *Pseudomonas*, *Arthrobacter*, *Alcaligenes*, *Corynebacterium*, *Flavobacterium*, *Achromobacter*, *Micrococcus*, *Mycobacterium*, and *Nocardia* are reported as the most active bacterial species in the degradation of hydrocarbons in soil (Bossert and Bartha, 1984). *Pseudomonas*, *Arthrobacter*, and *Achromobacter* often occur in greater numbers within rhizosphere soil than bulk soil (Walton et al. 1994). Soil fungi also play a role in the degradation of petroleum hydrocarbons. For example, a diversity of fungi, including *Aspergillus ochraceus*, *Cunninghamella elegans*, *Phanerochaete chrysosporium*, *Saccharomyces cerevisiae*, and *Syncephalastrum racemosum*, can oxidize various PAHs (e.g., anthracene, benz[*a*]anthracene, benzo[*a*]pyrene, fluoranthene, fluorene, naphthalene, phenanthrene, pyrene) as well as methyl-, nitro-, and fluoro-substituted PAHs (Sutherland, 1992). Radwan et al. (1995) investigated the microorganisms associated with various plants grown in soil polluted with 10% crude oil by weight. The plants used in the investigation included various Kuwaiti desert plants together with corn, tomato and termis. Rhizosphere samples of all plants were rich in oil-utilizing microorganisms.

2.4.1.3.2 Microbial degradation

Microbial degradation of organic contaminants normally occurs as a result of microorganisms using the contaminant for their own growth and reproduction. Organic contaminants not only provide the microorganisms with a source of carbon, they also provide electrons that the organisms use to obtain energy.

In general, the metabolic processes of microorganisms act on a wider range of compounds, carry out more difficult degradation reactions, and transform contaminants into more simple molecules than those of plants (Cunningham and Berti, 1993). However, not all microorganisms degrade organic contaminants in the same manner. The pathway of aerobic degradation of PAHs by prokaryotic microorganisms, such as bacteria, involves a dioxygenase enzyme, the incorporation of two atoms of molecular oxygen into the contaminant, and the production of less toxic compounds such as acids, alcohols, carbon dioxide and water (Gibson and Subramanian, 1984; Eweis et al. 1998; Pothuluri and Cerniglia, 1994). In contrast, degradation by eukaryotic fungi initially involves the incorporation of only one atom of oxygen into the PAH, which is similar to the degradation mechanism found in mammals (Sutherland, 1992; Cerniglia et al. 1986; Pothuluri and Cerniglia, 1994). Although most fungal transformations result in compounds that are less toxic than the parent PAHs, some of the minor metabolites produced during fungal degradation of PAHs result in compounds that are more toxic than the parent compounds (Sutherland, 1992).

There are several points of interest regarding microbial communities involved in the phytoremediation of organic contaminants. For example, the composition and size of the microbial community in the rhizosphere depends on plant species, plant age, and soil type (Campbell, 1985; Atlas and Bartha, 1998; Bossert and Bartha, 1984). The microbial community also may vary with exposure history; i.e., soil microbial communities may experience selective enrichment of contaminant-tolerant species when exposed to a contaminant for a prolonged period of time (Anderson et al. 1993). On the other hand, some species of bacteria can degrade a wide variety of rarely-occurring compounds without having to first adapt to contaminated conditions

(Siciliano and Germida, 1998). Catabolic pathways in *Pseudomonads*, for example, allow these bacteria to degrade a variety of aromatic contaminants (e.g., toluene, *m*-xylene, and naphthalene) without having to synthesize a large number of different enzymes (Houghton and Shanley, 1994).

2.4.1.3.3 Role of microorganisms in reducing phytotoxicity

Another role play by microbes involves their ability to reduce the phytotoxicity of contaminants to the point where plants can grow in adverse soil conditions, thereby stimulating the degradation of other, non-phytotoxic contaminants (Siciliano and Germida, 1998). In fact, Walton et al. (1994) have hypothesized that the defenses of plants to contaminants may be supplemented through the external degradation of contaminants by microorganisms in the rhizosphere. That is to say, plants and microbes have co-evolved a mutually-beneficial strategy for dealing with phytotoxicity, where microorganisms benefit from the plant exudates while the plants benefit from the ability of microorganisms to break down toxic chemicals.

Evidence in support of this hypothesis can be found in several studies. Improvement of rice grown in soil contaminated with oil residues resulted from the removal of the oil residues by various bacterial species of the genus *Bacillus*, which used plant exudates to cometabolize the oil residues in the rhizosphere. The root of *Senecio glaucus* plant grow along an oil leak polluted border of Kuwaiti desert and adhering sand particles were white and clean, while the surface of the transitional zone between the root and shoot was black and polluted (Radwan et al. 1995). The authors suggested that microbes detoxified contaminants in the rhizosphere, which allowed the plants to survive in the oil contaminated soils.

2.4.2 Containment

Containment involves using plants to reduce or eliminate the bioavailability of contaminants to other biota. Contaminants are not necessarily degraded when they are contained. Direct mechanisms of containment by plants include the accumulation of petroleum hydrocarbons within the plants and adsorption of the contaminants on the root surface. Another direct mechanism involves the use of plants as organic pumps to isolate the contaminant within the root zone, thus preventing the contaminant from spreading (Cunningham et al. 1996).

Plants act indirectly to contain contaminants by supplying enzymes that bind contaminants into soil organic matter in a process called humification and by increasing soil organic matter content, which allows for humification (Cunningham et al. 1996). For example, preliminary studies by Walton et al. (1994) suggest that ^{14}C originating from radiolabeled fluoranthene, phenanthrene and naphthalene may be incorporated by sweet clover (*Melilotus alba*) and its associated microorganisms into humic and fulvic acids found in the rhizosphere.

Several studies illustrate that plants take up petroleum hydrocarbons via their roots and may accumulate them to a small degree in their roots and shoots. Durmishidze (1977) reported that rice seedlings take up [^{14}C]methane through their roots and that bean and corn seedlings take up radiolabelled methane, ethane, propane, butane, and pentane through their roots and leaves. In experiments involving the fate of [^{14}C]benzene in soils planted with alfalfa, the result showed that 2% to 8% of the ^{14}C was recovered in the root fraction, which included small portions of rhizosphere soil attached to unwashed roots, and that less than 2% of the recovered 14C occurred in the shoots of plants (Ferro et al. 1997).

2.4.3 Transfer of petroleum hydrocarbons to the atmosphere

Soil may be phytoremediated by using plants to transfer volatile petroleum hydrocarbons from the soil to the atmosphere. In the phytoremediation literature, this process is also known as phytovolatilization (Flathman and Lanza, 1998). Wiltse et al. (1998) observed leaf burn in alfalfa plants growing in crude oil-contaminated soil. The authors suggested that an unidentified compound from the contaminated soil was being translocated through the plant and then transpired. The leaf burn gradually disappeared as the experiment progressed, indicating that the contaminants responsible for this effect had dissipated. Watkins et al. (1994) found that the volatilization of [^{14}C]naphthalene was enhanced in sandy loam soil planted to Bell Rhodes grass compared to unplanted soil. The results of the study suggested that naphthalene was taken up by the roots of the grass, translocated within the plant, and transpired through the stems and leaves. The authors noted that this mechanism of removal would reduce the amount of naphthalene available in soil, but may have implications regarding subsequent contamination of the atmosphere and, consequently, regulatory compliance with air quality guidelines.

2.5 Arbuscular mycorrhiza

2.5.1 Characteristics of arbuscular mycorrhiza

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Arbuscular mycorrhiza (AM) belong to the order Glomales and form highly branched structures called arbuscules, within root cortical cells of many herbaceous and woody plant species, are obligate endosymbionts that colonize plant roots of almost 90% of terrestrial plants. Another name, Vesicular-Arbuscular Mycorrhiza (VAM), comes from structures which are formed within root cortical cells, vesicles, which are thought to be storage or reproductive structures, and arbuscules, branch multiple-tipped hyphal

structures within the plant cell. Their composition is divided into 2 structure types including structures in root and soil.

Structures in roots composing of

Hyphae, non-septate when they are young and ramified within the cortex.

Arbuscules, intricately branched haustoria in cortex cells.

Vesicles, storage structures formed by many fungi.

Structures in soil composing of

Hyphae, a network of hyphae forms in the soil with thicker hyphae functioning as conducts and thin branched hyphae which are thought to absorb nutrients.

Spores, large asexual spherical structures (20-1000 μm diameter) that form on hyphae in soil or roots.

AM present in most natural and agricultural ecosystems are important for plant health, nutrient cycling, survival rate, and conservation of soil structure. AM procure and transport phosphate and other nutrients from the soil to plant roots. On the other hand, the host plant provides fixed carbon to its fungal partner (Harrison, 1999).

Furthermore, AM could facilitate the management of metal contamination in soil for a restoration and bioremediation program. This information indicated the challenge of the transferring this ability to non-legumes such as cereals representing a very long term or the possibilities of this fungus opening the sustainable agriculture. As a consequence, AM were crucial determinants of plant biodiversity, ecosystem variability, and the productivity of plant communities (Heijden et al. 1998).

ศูนย์วิทยทรัพยากร จุฬาลงกรณ์มหาวิทยาลัย

2.5.2 Classification of arbuscular mycorrhizal fungi

AM belong to Order Glomales which have taxonomy subsequence as summerized in Table 2.2.). Generally, we could find the AM form multinucleate spores in the soil which vary in wall structure and morphology between species and different stages of development (Walker and Trappe, 1993). At the present, some of these fungi could be identified on the basis of morphological features such as the spore surface,

spore color, spore shape, spore size, and spore ornamentation etc. However, these morphological variations make it difficult to identify the species presenting in natural ecosystems (Gioivannetti and Gianinazzi-Pearson 1994).

To ensure reliability in the identification of these fungi, analysis should be completed on spores from single-spore pot cultures or a large number of specimens collected from the field, in order to have a range of spores at different developmental stages and to find spores with mature cell walls which can be used for classification (Morton et al. 1994). However, this methodology is time consuming. The morphology-based identification of AM was limited when it was used in ecological settings because spore production was highly dependent on physiological parameters and may not be correlated with root colonization (Merryweather and Fitter, 1991). Therefore, using of molecular techniques represents a powerful tool for identifying and understanding the genetic variation of these organisms (Schwarzott and Arthur, 2001).

Table 2.2 Classification scheme for AM taxa (Morton and Benny 1990).

Order	Suborder	Family	Genus
Glomales	*Glominecae*	*Glomaceae*	*Glomus*
Glomales	*Glominecae*	*Glomaceae*	*Sclerocystis*
Glomales	*Glominecae*	*Acaulospora*	*Acaulosporaceae*
Glomales	*Glominecae*	*Archaeosporaceae*	*Entrophospora*
Glomales	*Glominecae*	*Archaeosporaceae*	*Archaeospora*
Glomales	*Glominecae*	*Paraglomaceae*	*Paraglomus*
Glomales	*Gigaspornea*	*Gigasporaceae*	*Gigaspora*
Glomales	*Gigaspornea*	*Gigasporaceae*	*Scutallospora*

2.5.3 Role of arbuscular mycorrhizal fungi and minerals uptake

When a nutrient is deficient in soil solution, the critical root parameter controlling its uptake is surface area. Hyphae of mycorrhizal fungi have the potential to greatly increase the absorbing surface area of the root. For example, Rousseau et al. (1994) found that while extramatrical mycelia (aggregates of hyphae) accounted for less than 20% of the total nutrient absorbing surface mass, they contributed nearly 80% of the absorbing surface area of the pine seedlings. It is also important to consider the distribution and function of the extramatrical hyphae. If the mycorrhiza is to be effective in nutrient uptake, the hyphae must be distributed beyond the nutrient depletion zone that develops around the root. A nutrient depletion zone develops when nutrients are removed from the soil solution more rapidly than they can be replaced by diffusion. For a poorly-mobile ion such as phosphate, a sharp and narrow depletion zone develops close to the root. Hyphae can readily bridge this depletion zone and grow into soil with an adequate supply of phosphorus. Uptake of micronutrients such as zinc and copper is also improved by mycorrhizae because these elements are also diffusion-limited in several soils (Jamal et al. 2002).

Another factor contributing to the effective absorption of nutrients by mycorrhizae is their narrow diameters relative to roots. The steepness of the diffusion gradient for a nutrient is inversely related to the radius of the absorbing unit; therefore, the soil solution should be less depleted at the surface of a narrow absorbing unit such as a hypha. Furthermore, narrow hyphae can grow into small soil pores inaccessible to roots or even root hairs. Another advantage attributed to mycorrhizal fungi is access to pools of phosphorus not readily available to the plant. One mechanism for this access is the physiochemical release of inorganic and organic phosphorus by organic acids through the action of low-molecular-weight organic anions such as oxalate which can be: (i) replace phosphorus sorbed at metal-hydroxide surfaces through ligand-exchange reactions, (ii) dissolve metaloxide surfaces that absorbs phosphorus, and (iii) complex metals in a solution and thus prevent precipitation of the metal phosphates (Fox et al. 1990).

2.5.4 AM formation

In nature, most plants do not only have roots; instead they have mycorrhizae, the symbiotic association of a fungus and plant roots. AM are the most common root endosymbiotic association, and are formed between the roots of most higher plants and fungi. AM fungi are obligate biotrophs and strictly dependent on their host plant for survival. As with the *Rhizobium*–legume interaction, this symbiosis is set in motion by the exchange of signals between the two symbionts, although the nature and the mechanism of action of these molecules are unknown. Exudates from a host root, especially (iso) flavonoids, enhance spore germination, and elongation and branching of hyphae (Giovannetti et al. 1993). At the root surface the hyphae form swollen structures, named appressoria. The formation of appressoria is initiated upon contacting the cell wall of a root epidermal cell. In contrast, appressoria are not formed when contacting cortical or vascular cell walls, indicating that the fungus recognizes specific epitopes present in the cell wall of root epidermal cells (Nagahashi and Douds, 1997).

The appressoria become firmly attached to the root epidermis and subsequently new hyphae develop which will enter the root. Depending on the host plant this can occur either intercellularly or intracellularly. Since AM involving intercellular infection, the Arum type, is found predominantly in cultivated herbs, it has become more frequently studied than the AM involving intracellular infection, the Parish type (Smith and Smith ,1997). The plant accommodates the invasion of the fungus by secreting new cell wall material which surrounds the infecting hyphae. In the inner cortex, the fungus invades cells and there they differentiate into highly ramified structures, the arbuscules. These structures are thought to facilitate the exchange of nutrients between both organisms.

Although arbuscules occur intracellularly, they are never in direct contact with the cell cytoplasm. A perifungal membrane, originating from the plant plasma membrane invaginates and surrounds the arbuscules. During the formation of arbuscules, the plant cell becomes cytoplasmically dense, its vacuole fragments, and the number of Golgi bodies increases. Furthermore, the nucleus moves to a more central position in the cell (Balestrini et al. 1992).

When the mycorrhizal fungi colonize the roots and appressoria have been formed, the fungus rapidly enters the cortex. Upon entry the root arbuscules are formed within a few days. Arbuscules have a similar morphology as haustoria; the feeding structures which are formed by several pathogenic fungi during a compatible interaction. During both haustorium and arbuscle formation, plant defense responses are induced, but only at a low level. For these reasons it seems probable that haustorium and arbuscle formation involve similar mechanisms. In contrast to the *Rhizobium*–legume interaction, there is very little host specificity in AM symbioses. A fungus can interact with a diverse range of host plant species, whereas a certain host plant can interact with several fungal species. However, several plant families can be considered as non-mycorrhizal or rarely mycorrhizal, e.g. the *Brassicacea.* (Albrecht et al. 1998)

2.5.5 AM and plant symbiosis

AM associations form when host roots and compatible fungi are both active in close proximity and soil conditions are favourable. The infection process consist of three different steps: (1) hyphal growth from a germinating spore, dependent initially upon its own nutritional supply; (2) stimulation of further fungal growth by root exudates and initiation of the infection process; and (3) fungal development of intracellular arbuscules which connect the fungus to the nutrient flux from the plant (Giovannetti et al. 1993).

2.5.5.1 Soil hyphae

Mycorrhizal associations were initiated by spore germination. Hyphae were also originated from fragments of roots. In many cases, there is already a pre-existing network of hyphae resulting from previous root activity. Hyphae resulting from spore germination have a limited capacity to grow and will die if they do not encounter a susceptible root within a week or more (Gianinazzi-Pearson,1996).

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

2.5.5.2. Root contact and penetration

Mycorrhizal associations start when soil hyphae respond to the presence of a root by growing towards it, establishing contact and growing along its surface. Next, one or more hyphae produce swellings called appressoria between epidermal cells. Root penetration occurs when hyphae from the appressoria penetrate epidermal or cortical cells to enter the root. These hyphae cross the hypodermis (through passage cells if these are present in an exodermis) and start branching in the outer cortex.

2.5.5.3. Arbuscules

Arbuscules are intricately branched haustoria that formed within a root cortex cell because they look like little trees. Arbuscules are formed by repeated dichotomous branching and reductions in hyphal width, starting from an initial trunk hypha (5-10 um in diameter) and ending in a proliferation of fine branch hyphae (< 1 um diameter). Arbuscules start to form approximately 2 days after root penetration. They grow inside individual cells of the root cortex, but remain outside their cytoplasm, due to invagination of the plasma membrane. Arbuscules are considered the major site of exchange between the fungus and host. This assumption is based on the large surface area of the arbuscular interface, but has not been confirmed (Smith 1995). Arbuscule formation follows hyphal growth, progressing outwards from the entry point. Arbuscules are begun to collapse after a few days, but hyphae and vesicles can remain in roots for months or years.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

2.5.5.4. Vesicles

Vesicles were developed to accumulate storage products in many VAM associations. Vesicles are initiated soon after the first arbuscules, but continue to develop when the arbuscules senesce. Vesicles are hyphal swellings in the root cortex that contain lipids and cytoplasm. These may be inter- or intracellular. Vesicles can

develop thick walls in older roots and may function as propagules (Biermann and Linderman 1983). Some fungi produce vesicles which are similar in structure to the spores they produced in soil, but in other cases they are different.

2.5.5.5. Spores

Spores form as swellings on one or more subtending hypha in the soil or in roots. These structures contain lipids, cytoplasm and many nuclei. Spores usually develop thick walls with more than one layer and can function as propagules. Spores may be aggregated into groups called sporocarps. Sporocarps may contain specialised hyphae and can be encased in an outer layer (peridium). Spores apparently form when nutrients are remobilised from roots where associations are senescing. They function as storage structures, resting stages and propagules. Spores may form specialised germination structure, or hyphae may emerge through the subtending hyphae or grow directly through the wall.

2.6 Rhizobium-induced nodule formation

2.6.1 Rhizobium

Most higher plants have the ability to form arbuscular mycorrhiza (AM), a symbiotic association of the plant root with fungi belonging to the order of *Glomales*. In contrast to AM formation, only a few plant species have the ability to interact symbiotically with bacteria of the genera *Azorhizobium*, *Bradyrhizobium*, *Rhizobium* and *Sinorhizobium*. This interaction is almost completely restricted to leguminous plants and results in the formation of a completely new organ, the root nodule. In these nodules the bacteria are hosted intracellularly and there they find the ideal environment to reduce atmospheric nitrogen into ammonia, a source of nitrogen which can be used by the plant (Long, 1996).

2.6.2 Nod factors

Root-nodule formation involves growth responses in the epidermis as well as cortex of the root. This implies that the bacteria redirect the development of fully differentiated plant cells. The bacterial signals that set this in motion are the so-called nodulation (Nod) factors. Nod factors of the different *Rhizobium* species have a common basic structure; a β-1,4-linked *N*-acyl-D-glucosamine backbone of mostly four or five units, containing a fatty acid at the non-reducing terminal sugar (Long, 1996).

The biosynthesis of the basic Nod-factor structure is catalysed by the bacterial NodA, NodB and NodC proteins. NodC is an *N*-acetylglucosaminyl-transferase and catalyses the synthesis of the chitin oligomer and controls the length of this backbone. The terminal non-reducing glucosamine unit of this oligomer is deacetylated by NodB, and subsequently substituted with an acyl chain by NodA. Several other Nod proteins, which can be specific for a certain *Rhizobium* species, modify a terminal sugar residue or determine the nature of the acyl chain (Carlson et al. 1994). These modifications define the biological activity and host specificity of Nod factors. As an example, Nod factors produced by *Sinorhizobium meliloti* (previously named *Rhizobium meliloti*) and *Rhizobium leguminosarum* biovar *viciae*, are shown in Figure 2.1. The major difference between both Nod factors concerns the presence of a sulfate group at the reducing terminal sugar of the *S.meliloti* factor and the structure of the acyl chain.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

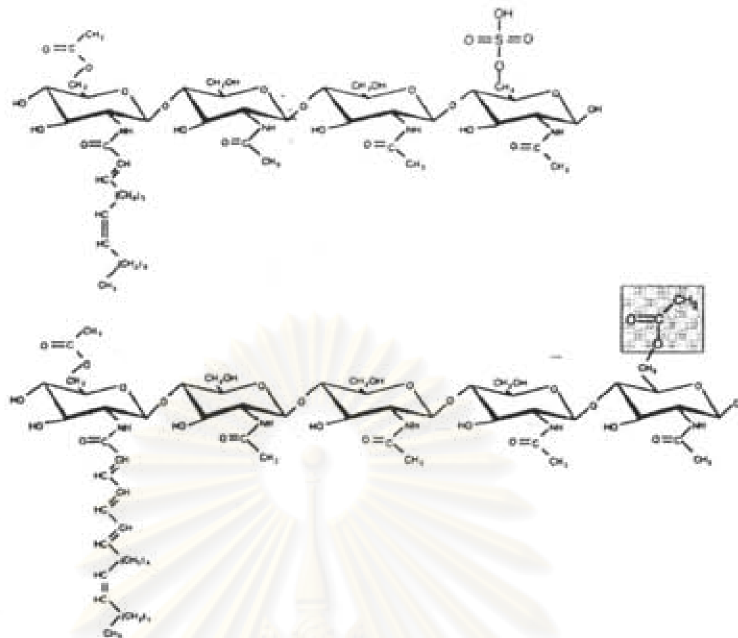


Figure 2.1 Nod factors produced by *Sinorhizobium meliloti* and *Rhizobium leguminosarum* biovar *viciae* (Lerouge et al. 1990).

Since bioactivity of Nod factors is controlled by their structure. It is very likely that they are recognized by receptors of the host. However, such receptors have not been cloned. Biochemical studies have shown that a few Nod-factor binding proteins occur, but it is not yet clear whether these are Nod-factor receptors (Niebel et al. 1997).

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

2.6.3 <u>Nodulation process</u>

Nodulation process Nod-factor-secreting rhizobia induce shepherd's crook-like curling of root hairs within 1 2 days of inoculation. Rhizobium uses the microenvironment within such curls to establish an infection site. They locally degrade the plant cell wall and enter the root hair via invagination of the plasma membrane (Turgeon and Bauer, 1985). Vesicles are directed to the invaginated membrane, leading to the formation of an inward tip growing tubular structure, the infection thread. In

general, Nod factors are not sufficient to trigger root-hair curling and infection-thread formation, but they play a crucial role in the infection process, since infections can only be initiated when the bacteria secrete specific Nod factors (Geurts et al. 1997).

Results obtained with bioassays have provided some insight into the mechanism by which Nod factors alter the growth pattern of root hairs. Such studies have most extensively been carried out using vetch (*Vicia sativa*). Vetch-root hairs which respond morphologically to the application of Nod factor have almost terminated growth. The morphological changes start with swelling of the roothair tip, which occurs within 1 hour of Nod-factor application (Heidstra et al. 1994). This swelling is the result of isotropic growth, is accompanied by the formation of a calcium gradient at the plasma membrane and requires protein synthesis (Vijn et al. 1995). At these swollen tips, new tip growth is initiated and the cyto-architecture of the resulting outgrowth shows a strong resemblance to that of normal growing root hairs. Such studies show that Nod factors can re-induce (tip) growth in root hairs. However, it remains unclear how Nod-factor secreting bacteria can redirect tip growth in such way that shepherd's crook-like curls are formed. Furthermore, whether the bacteria exploit and modify this growth process for infection-thread formation remains to be resolved.

Nod-factor-induced growth responses in root hairs are preceded by rapid physiological changes. These involve a rapid influx of calcium into the hairs. Shortly after this calcium flux, an opposite-directed flux of chloride ions occurs, which is accompanied by a depolarization of the root-hair membrane (Felle et al. 1998). These processes are followed by an alkalinization of 0.2 0.3 pH units of the root-hair cytoplasm (Felle et al. 1996). Several minutes after the application of Nod factors, a regular oscillation of cytoplasmic calcium occurs around the nucleus (Ehrhardt et al. 1996). Whether these physiological changes are involved in the alteration of growth of hairs is unknown.

Nod factors can mitotically activate clusters of cortical cells by which nodule primordia are formed. Which cortical cells will form a nodule primordium is determined by the host plant. Primordia are mainly formed opposite the protoxylem poles and furthermore, the host species determines whether inner or outer cortical cells are

involved in primordium formation. When the nodule primordia are formed in the outer cortical cell layers, as in soybean (*Glycine max*), the infection thread grows through the root hair and can immediately invade the primordium. In contrast, in legumes, e.g. pea (*Pisum sativum*) and *Medicago* species, in which nodules are formed in the inner cortex, the infection thread has to cross several cortical cell layers before reaching the primordium. The cortical cells which will be traversed by an infection thread re-allocate their nuclei to their centre from where microtubules and cytoplasmic strands are positioned anticlinically to the advancing infection thread (Van Brussel et al. 1992). By using cell-cycle phasespecific markers it has been shown that these cells have entered the cell cycle but become arrested in G_2, which indicates that the cytological structure found resembles a phragmoplast (Yang et al. 1994). A radial array of cortical cells containing such phragmoplast-like structures provides a track for the infection thread to support its growth and to guide it to the primordia. The formation of phragmoplast-like structures during infection shows that rhizobia recruit and modify a common process, namely cell division, and use this for a completely different purpose, the infection process.

When the infection thread reaches the primordium, the bacteria are released, and enter the cytoplasm via an invagination of the host-cell membrane. Within the host cytoplasm the bacteria remain surrounded by a host membrane, and together they form a so-called symbiosome that divides. In this way the bacterial surface is never in direct contact with the plant cytoplasm. Upon infection the nodule primordia simultaneously form a meristem as well as the different tissues that form a nodule. In most species nodules are macroscopically visible 4 7 days after inoculation. The meristems maintain their mitotic activity, at least during a substantial part of the lifetime of a nodule, and they add cells to the different nodule tissues by which the organ grows.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

2.6.4 Host specificity

Nod-factor-controlled host specificity, an intriguing property of the *Rhizobium*–legume interaction is its host specificity. Most rhizobia have a very narrow host range. For example, *S. meliloti* is able to interact with *Medicago*, e.g. alfalfa (*Medicago sativa*), *Trigonella* and *Melilotus* species, whereas *R. leguminosarum* bv *viciae* forms nodules on species of the genera *Pisum* (e.g. pea), *Vicia* (e.g. vetch), *Lathyrus* and *Lens*.

In most cases host specificity is controlled at several levels, but synthesis and the structure of Nod factors often play a prominent role. The rhizobial genes encoding the enzymes involved in the biosynthesis of Nod factors are activated when the bacteria grow in the vicinity of the root system of their host plants. There, rhizobia sense signal molecules, which are usually flavonoids, secreted by the host. These molecules activate the constitutively formed transcriptional regulators, NodD, that induces the expression of the other Nod genes of which most are involved in the biosynthesis and secretion of Nod factors. The nature of the flavonoids secreted by the host can play a key role in controlling host specificity. For example, *Rhizobium etli* and *Rhizobium loti* secrete Nod factors with an identical structure, but their NodDs are only activated by elicitors secreted by their specific host plant. By introducing a gene encoding a constitutively active NodD protein these bacteria obtained a broader host range and could infect each other host plant (Cardenas et al. 1995).

The length of the glucosamine backbone, the structure of the acyl chain and specific substitutions at the terminal sugar residues of the produced Nod factors can all contribute to the ability of the bacterium to nodulate their host plants. Changes in Nod-factor structure, either by Nod gene mutations or introduction of Nod genes of other *Rhizobium* species, mostly results in a decreased nodulation potential of the strain, but in some cases it obtains the ability to nodulate non-host plants. For example, mutating the sulfotransferase-encoding gene *nodH* of *S.meliloti*, which is involved in sulfation of the reducing sugar terminus of the Nod factor (Roche et al. 1991), results in the inability of such strains to interact with alfalfa, but it has gained the ability to interact with vetch (Faucher et al. 1989). Such experiments demonstrate that the plant discriminates the

different *Rhizobium* species by recognizing their specific Nod factors. Some rhizobia have overcome this restriction by producing a great variety of Nod factors, e.g. *Rhizobium* sp. NGR234 (Price et al. 1992), which can nodulate a broad host range encompassing legume species of more than 110 genera (Freiberg et al. 1997) as well as the only non-legume known to establish a symbiosis with rhizobia, *Parasponia andersonni*.

Strict regulation of bacterial entry of all Nod-factor-controlled responses, bacterial entry appears to be the most stringently controlled. Infection-thread initiation growth in the root epidermis will only occur efficiently when the rhizobia produce Nod factors with a specific structure, whereas other responses depend less on Nod factor structure. For example, *S.meliloti* strains mutated in either *nodL*, leading to an absence of the *O*-acetylation of the non-reducing terminal sugar residue, or mutated in *nodFE*, leading to the absence of specific unsaturated bonds in the acyl chain, are both seriously hampered in the infection process, whereas other Nod-factor-induced plant responses are not affected (Ardourel et al. 1994).

Alternatively, it is possible that Nod factors can induce the infection response at variable levels depending on the structure of the Nod factor. This would imply that the ability of the plant to block the growth of infection threads is lower when the infection response is higher.

Besides Nod factors, other components can also facilitate infection-thread growth. For several plants it has been shown that deficiencies in Nod-factor structure, by which infections are hampered, can be complemented, in part, by the rhizobial NodO protein (Vlassak et al. 1998). NodO is a secreted protein, which is not involved in Nod-factor production or secretion. It has been shown that NodO is able to bind calcium and it can integrate into artificial membranes where it forms ion channels (Sutton et al. 1994). Therefore, it has been postulated that it will form ion channels in the host plasma membrane as well, where it could contribute to the suppression of the incompatibility mechanism or the induction of the infection responses.

Other host proteins involved in the regulation of infection-thread growth are lectins. In the root, lectins are present in relatively low amounts and are localized on the

external surface of elongated epidermal cells and on the tips of developing root hairs (Van Rhijn et al. 1998). Introduction of the pea lectin into white clover (*Trifolium repens*) showed to increase nodulation by its host strain *R.leguminosarum* bv *trifolii* (Diaz et al. 1995). Strikingly, expression of heterologous lectins also facilitates infection by non-host rhizobia, showing that lectins—in analogy to NodO—decrease the threshold level for the infection response (Diaz et al. 1995). How this is achieved is not exactly known, however, data obtained with a lectin mutated in the carbohydrate-binding site shows that this protein is unable to extend the host range and can also no longer facilitate attachment of the bacterium to the root-hair surface (Van Rhijn et al. 1998).

2.7 Interaction of soil microorganisms

2.7.1 AM-rhizobacteria interaction

The increased microbial activity in the rhizosphere soil affects the plant. A range of stimulated rhizosphere microorganisms such as saprophytes, pathogens, parasites, symbionts, etc., carry out many activities which are important to plant health and growth. Some of these microbes affect plant root morphology and physiology by producing plant growth-regulating hormones and enzymes. Others alter plant nutrient availability and biochemical reactions undertaken by them. AM fungi have differential effects on the bacterial community structure in the mycorrhizosphere (Marschner and Baumann, 2003).

AM improve P-nutrition by scavenging available P through the large surface area of their hyphae. Plant growth promoting rhizobacteria, PGPR, may also improve plant P-acquisition by solubilizing organic and inorganic P sources through phosphatase synthesis or by lowering pH of the soil (Rodríguez and Fraga, 1999). Garbaye (1994) defined MHB (mycorrhization helper bacteria) as bacteria associated with mycorrhizal roots and mycorrhizal fungi which collectively promote the establishment of mycorrhizal symbioses. There is growing evidence that diverse microbial populations in the

rhizosphere play a significant role in sustainability issues and that the manipulation of AMF and certain rhizobacteria like PGPR and MHB is important. Vivas et al. (2003) used a dual AM fungus-bacterium inoculum to study the effect of drought stress induced in lettuce grown in controlled-environment chambers. Their results showed a specific microbial-microbial interaction that modulates the effectivity of AMF on plant physiology. The authors concluded that plants must be mycorrhizal in nutrient-poor soils and that mycorrhizal effects can be improved by co-inoculation with MHB such as *Bacillus* sp.

Co-inoculation of selected free-living bacteria isolated from adverse environments and AM fungi can improve the formation and function of AM symbiosis, particularly when the plant growth conditions are also adverse (Vivas et al. 2003). Both AMF and PGPR complement each other in their role in N-fixation, phytohormone production, P-solubilization, and increasing surface absorption. Chaudhry and Khan (2003) studied the role of symbiotic AMF and PGPR N-fixing bacterial symbionts in sustainable plant growth on nutrient-poor heavy metal contaminated industrial sites and found that the plants surviving on such sites were associated with N-fixing rhizobacteria and had a higher arbuscular mycorrhizal infection, i.e. a cumulative and synergistic effect. The MHB cannot be ignored when studying mycorrhizal symbioses in their natural ecosystems. They are quite common and found every time they are looked for, and they seem to be closely associated with the mycorrhizal fungi in the symbiotic organs. They are adapted to live in the close vicinity of the AM fungi as high frequencies of MHB populations are isolated from the mycorrhizae. Some MHB isolates also promoted ectomycorrhizae formation in four conifers (Garbaye et al. 1992), indicating that the MHB effect is not plant-specific.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Various researchers showed that MHB's are fungus selective. Mayo et al. (1986) showed that cell-wall degrading enzyme producing *Pseudomonas* sp. enhanced the germination of AM fungal spores of *Glomus mosseae* and promoted the establishment of AM on clover roots under aseptic conditions. Microbial colonies on the root surfaces consist of many populations or strains and positive and negative inter-population signaling on the plant root occur (Pierson et al. 2002), which may play an important role in the efficiency of the use of biofertilizers.

2.7.2 Plant growth promoting rhizobacteria

Rhizobacteria include mycorrhization helper bacteria (MHB) and plant growth promoting rhizobacteria (PGPR), which assist AM to colonize the plant roots (Andrade et al. 1997), P-solubilizers, free-living and symbiotic nitrogen fixers, antibiotic producingrhizobacteria, plant pathogens, predators and parasites. The most common bacteria inthe mycorrhizosphere are *Pseudomonas*, while different bacterial species exist in the hyphosphere. Like AM, rhizobacteria such as *Pseudomonads*, are also ubiquitous members of the soil microbial community and have received special attention as they also exert beneficial effect on plant growth by suppressing soil-born pathogens, synthesizing phytohormones, and promoting plant growth (Glick, 1995 and Chin-A-Woeng et al. 2003).

Many fluorescent *Pseudomonas* strains have been reported as plant growth enhancing beneficial rhizobacteria. They are studied extensively in agriculture for their role in crop improvement as they stimulate plant growth either by producing plant growth promoting hormones, fixing atmospheric N2 or suppressing plant pathogens. Rhizospheric component of PGPR bacteria adher to the root surfaces, use root exudates for growth, synthesize amino acids and vitamins, and establish aneffective and enduring root colonization (Lugtenberg and Dekkers, 1999). However, quantitative and qualitative variations in root exudates during plant growth could affect the rhizospheric competency of introduced PGPRs. Many researchers have reported additive effects on plant growth by AM and PGPR but the mechanisms by which MHB and PGPR stimulate AM colonization are still poorly understood (Barea et al. 1997). Various hypotheses have been suggested which include physical, chemical, physiological, and even direct stimulatory or antagonistic relations between AM and other mycorrhizosphere microbes.

AM and PGPR symbioses not only induce physiological changes in the associated plant, but also modify the morphological architecture of the roots such as total root length and root tip numbers (Atkinson et al. 1994). PGPR have also been shown to induce systemic resistance to fungal, bacterial, and viral pathogens in various crops such as bean, tomato, radish, and tobacco (Zhang et al. 2002)

2.7.3 AM-rhizobial interaction

Common host genes are involved in both the rhizobial and mycorrhizal interactions. This finding has an important implication since in contrast to Rhizobium, AM fungi have the ability to interact with a wide range of higher plants. Assuming that the mechanisms by which AM fungi infect their various hosts are sīmilar, it implies that genes required for the interaction of legumes with both micro-symbionts, are most probably widespread in the plant kingdom.

Co-inoculation of AM fungi, *Glomus coronatum*, with *Rhizobium* sp. was also studied and was the most effective treatment for improving N content. The role of AM fungi as P suppliers to the plant, and particularly to the nodule, appears to be of great relevance. The symbiotic nitrogen fixation results were favoured by the increased P content in plant tissues due to the AM benefits the uptake of immobile ions. Thus increases in P content of plants inoculated with *Rhizobium* sp. and *Glomus coronatum* cand be also associated with higher N contents due to increase of N fixation (Requena et al. 1997).

2.7.4 AM and rhizobium induced phytoremediation of contaminated

The efficacy of AM, rhizobium, and plant interaction was assessed to increase the rate at which microorganisms and plant remove contaminated soil. Chaineau et al. (1997) observed no uptake of petrogenic hydrocarbons at contamination levels not inhibiting maize (*Zea mays* L.) growth. In heavily contaminated soil, however, adsorption of hydrocarbons to leaves and stems was rapid and maize plants died quickly. Radwan et al. (2000) found that long chain hydrocarbons accumulated in broad bean (*Vicia faba*), grown in oil contaminated soil. The accumulation, especially into seeds, was thought to pose a risk to human or animal nutrition. Hydrocarbons were also found in poplar and pine root extracts. Planted soils augmented with bacteria could benefit PAH degradation. The degree of anthracene degradation in the soil also appeared to differ between microbial species. The bacterium *S. paucimobilis* was effective in reducing

anthracene in soil compared with *P. aeruginosa*. The introduced bacteria generally could maintain their viability and activity in the presence of anthracene. Electrofused bacteria used in their study were capable of degrading anthracene and survived well in soil (Shinkarev et al. 1999).

In addition, the phytoremediation of soils contaminated by heavy metals had been studied, an ideal plant species for remediation purposes should grow easily on soils contaminated by metals, have high soil-to-shoot transfer factors, tolerate high shoot metal concentrations, and produce high biomass quickly (Banuelos et al. 1997).

2.8 Characterizations

2.8.1 <u>Gas chromatography</u> (Schwab et al. 1999)

Gas chromatography (GC) is a dynamic method of separation and detection of volatile organic compounds and has chosen as an attractive method to analyze the complex mixture of diesel oil. GC involves the partitioning of gaseous solutes between an inert carrier gas as mobile phase and a stationary liquid or solid phase. Figure 2.2 is the major compartments of GC consist of gases, injection port, column, detector, and data acquisition system.

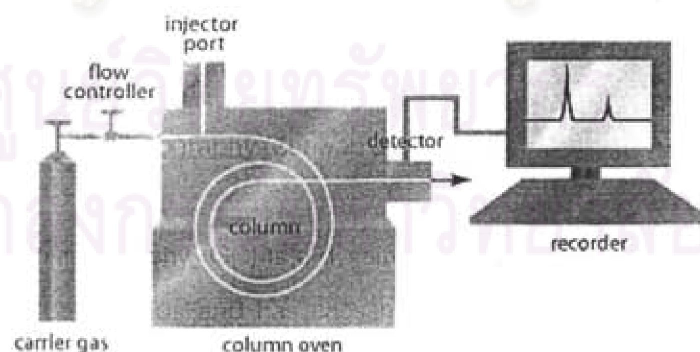


Figure 2.2. Gas chromatograph compartments.

Helium is one of the most commonly used gases to carry the sample through the system. Increase the flow rate of carrier gas results in shortens the retention time. Injection port is the next major component to introduce the sample into the carrier gas stream. A split injection port is designed to allow only small fraction of sample volume into the column because of a limited sample capacity of the capillary column. Many split injection port can also operate in a splitless mode. In the splitless mode most of the sample is allowed to the column. This technique is suitable for samples with trace concentration. The third major component is column for separation of the components in the sample mixture. The more narrow the diameter and length, the greater the column efficiency to separate sample. The fourth major component of GC is the detector that senses the presences of components separated from the column and convert that information to the signal. The most commonly used detector is flame ionization detector (FID) because it responds to any type of hydrocarbon component. When hydrocarbon components burn in a flame, producing ions that are collected and converted into a current signal. As the components are separated and reach the detector, the signal increases and the detection or retention time of each peak is recorded and translated into a chromatogram by data acquisition system.

Gas chromatography is a well-established method in the analysis of hydrocarbon products. Identification of gas chromatography is based either on the comparison with known chromatograms or the detailed analysis of products such as the combination with mass spectrometry.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

2.8.2 Gas chromatography-mass spectrometry (Siu et al. 2005)

Gas chromatography can separate volatile compounds with great resolution, but it cannot identify them. So mass spectrometry (MS) coupled with GC can provide detailed structural information of separated compounds.

In the first part of the MS, the ionizer which is an electron beam causes the components to gain a positive charge. This process causes further breakdown of the individual components. Each component has a unique fragmentation pattern. The sub-

components then enter a magnetic region where they are focused and sent to a detector. Components with a lighter atomic mass enter the magnet set up and will be sent to the detector first. At the detector, the component transfers their charges which then activates a recorder capable of registering atomic mass, based on the mass/charge ratio, and identify the concentration of the particular component in the sample.

CHAPTER III

MATERIALS AND METHODS

3.1 Arbuscular mycorrhiza isolation and inoculums preparation

Arbuscular mycorrhiza (AM) fungi were collected from various soil and plant roots (*Acacia mangium* Willd., *Glycine max* (L.) Merr., *Vigna radiate (L.)* Wilczek, *Sorghum bicolor (L.)* Moench, *Zea mays* L., *Allium ascalonicum* L., *Brachiaria ruziziensis* R. Germ. & C. M. Evrard, *Morus alba* L., *Tectona grandis* L.f., *Tamarindus indica* L., *Parkia speciosa* Hassk., *Poinciana pulcherrima* L., *Saccharum officinarum* L., and *Carica papaya* L.) in seven provinces of Thailand as shown in Table 3.1. One kg of each soil was randomized sampling at the depth of 0 – 30 cm and used for arbuscular mycorrhiza isolation.

3.1.1 <u>Wet sieving and decanting technique</u>

AM spores were isolated from 200 g of each soil sample using wet sieving and decanting technique (Gerdemann and Nicolson, 1963). Approximately 200 g of soil were suspended in one liter of water. Heavier particles were allowed to settle for a few seconds and the liquid was decanted through 750, 250, 100 and 45 μm sieve fine enough to remove the larger particles of organic matter, but coarse enough to allow the desired spores to pass through. The suspension that passed through these sieves was saved and stirred to resuspend all particles. The heavier particles were allowed to settle for a few seconds and the liquid was decanted again through the sieve, ensure that soil aggregates have broken apart. This washing and decanting process was repeated until the water was clear. Vigorous washing with water and hand mixing were necessary to free spores from aggregates of clay or organic materials. The sievings retained on

different sieves were washed into separate petri dishes for further observations or purification by sucrose centrifugation.

Table 3.1 Plant species and sampling site for AM collection

Plant species	Sampling site
Acacia mangium Willd.	Nakonratchasima
Glycine max (L.) Merr.	Nakonratchasima
Vigna radiate (L.) Wilczek	Nakhonsawan
Sorghum bicolor (L.) Moench	Nakhonsawan
Zea mays L.	Yasothon
Allium ascalonicum L.	Yasothon
Brachiaria ruziziensis R. Germ. & C. M. Evrard	Khonkaen
Morus alba L.	Khonkaen
Tectona grandis L.f.	Phetchaburi
Tamarindus indica L.	Phetchaburi
Parkia speciosa Hassk.	Lopburi
Poinciana pulcherrima L.	Lopburi
Saccharum officinarum L.	Bangkok
Carica papaya L.	Bangkok

3.1.2 Sucrose centrifugation

Sieving particles from sieves were purified by sucrose centrifugation to separate AM spores (Daniel and Skipper 1979). Spores and minimal amount of organic particles could be further purified by suspending sievings in the 40% sucrose solution and centrifugation at 2000 rpm (approximate 370 x g) for 1 minute to separate spores from denser soil components. The supernatant (with spores) was poured through the sieve of 45 μm and rinsed with distilled water to remove sucrose.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

The isolated AM spores were soaked in 2 % chloramin T mixing with 0.05 % Tween 20 for 20 min. Spores were washed 3 times in sterile distilled water then soaked in 200 ug/ml streptomycin and 100 ug/ml gentamycin for 20 min.

3.1.3 Inoculum preparation

Peat mosses were used as plant substrate. They were sterile twice by autoclaving at 100°C, 15 psi for 1 hour. Plastic pots used for planting were cleaned by 70% ethylalcohol. A disinfected AM spore was transferred to a sterile pot culture containing seedlings of *Sorghum bicolor* and grew for 3 months (Figure 3.1).

Figure 3.1. A three-month-old pot culture of *Sorghum bicolor*.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

3.1.4 Isolation and propagating of AM

Pot culture isolation procedures help to identify AM from soils collected in the field that didn't contain spores of sufficient quantity to be accurately identified. The isolated AM spores were propagated by growing with *Sorghum bicolor* pot cultures. These pot cultures consisting of soil, spores, root pieces and hyphal fragments were used as inoculum for the experiments.

The root of *Sorghum bicolor* was sampling for root infection following Philips and Hayman (1970) method. AM structures could be observed by the use of stain which bind to hyphae without much background staining. Trypan blue in lactoglycerol was used to stain AM structures in roots that had been cleared by heating in KOH. The procedure was outlined as follow,

1. Cut roots into 2 – 4 cm long segments before staining for uniform contact with solution.
2. 10% w/v KOH was used to clear roots by autoclaving at 121°C for 20 minutes.
3. Cleared roots were captured on a fine sieve and rinsed with water before transferring them into the staining solution.
4. Stained with 0.05% w/v of trypan blue in lactoglycerol at 121°C for 15 minutes in autoclave.

Spore production was determined from soil using gridline intersection method. The AM isolate that exhibited the most root infection and sporulation was selected as an inoculum in the experiment. Inoculum preparation was done by cutting the whole plant above ground off then the remained roots and soil were kept at 4 °C prior to use.

3.1.5 Identification of selected AM fungi

To obtain the information for basic identification, the specimen slides were prepared according to the method of Koske and Gemma (2000). After isolate the spores from fresh pot culture, a minimum of 20 spores per each host plant were collected in glass watch. These spores were analyzed in both forms of intact and crushed after mounted with PVLG (polyvinyl lactoglycerol) and Melzer's PVLG (INVAM, 1997). The criterion of genus identification was summarized using Taxonomy and identification of arbuscular mycorrhizal fungi in genera level (Bernhard, 2002)

After isolation and identification of AM from each sampling soil, the *Glomus mosseae* was the most AM found. Then *Glomus mosseae* was propagated in *Sorghum bicolor* for 3 months to investigate the number of spore from each soil. The *Glomus*

mosseae isolate that exhibited the highest number of spores and %AM infection derived from *Acacia mangium* Willd. sampling in Nakonratchasima.

3.2 Rhizobium preparation

Rhizobium fertilizer was obtained from Department of Agriculture, Thailand.

3.3 Biostimulation by AM and rhizobium

The seeds of Jack bean (*Canavalia* sp.) were surface sterilized by 0.5 N sulfuric acid for 12 hr then soaked in 5% sodium hypochlorite for 5 min, rinsed with sterile distilled water 3 times then re-soaked one min in 70% ethylalcohol and rinsed 3 times with sterile distilled water.

Peat mosses were used as plant substrate. They were sterile twice by autoclaving at 100°C, 15 psi for 1 hour. Plastic pot used for planting was cleaned by 70% ethylalcohol.

The biostimulation of Jack bean was conducted using AM spores and rhizobium in green house. The experiment consisted of soil pots amended with AM at 10, 20, 50, or 100 spores or 10 g rhizobium or the mixture of both microorganisms (Table 3.2). The control treatment consisted of Jack bean pots without any amendment.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Table 3.2 Detailed amendment of each biostimulation experiment

Experiment	Treatment*	Jack bean	Rhizobium fertilizer (g)	AM (spores)
1	P	✓	-	-
2	R+P	✓	10	-
3	A10+P	✓	-	10
4	A20+P	✓	-	20
5	A50+P	✓	-	50
6	A100+P	✓	-	100
7	R+A10+P	✓	10	10
8	R+A20+P	✓	10	20
9	R+A50+P	✓	10	50
10	R+A100+P	✓	10	100

* P: plant without inoculation, R+P: 10 g rhizobium with plant, A10+P: 10 spores AM with plant, A20+P: 20 spores AM with plant, A50+P: 50 spores AM with plant, A100+P: 100 spores AM with plant, R+A10+P: 10 g rhizobium and 10 spores AM with plant, R+A20+P: 10 g rhizobium and 20 spores AM with plant, R+A50+P: 10 g rhizobium and 50 spores AM with plant, R+A100+P: 10 g rhizobium and 100 spores AM with plant

Complete randomized design (CRD) of 10 pots with 3 replicates of each soil treatment was setup. The study was conducted in greenhouse with daily watering and Bell's nutrient solution (detail in index) was weekly added.

Three months after inoculation, plant growth, biomass, and % AM infection on root of plants were evaluated. Plant growth has been evaluated by height and stem diameter. Biomass was determined by plant dry matter obtained by heating at 40 °C for 12 hr. Roots were rinsed 5 times with water before drying then %AM infection on root was conducted following Philips and Hayman (1970) method.

3.4 Phytoremediation

Jack bean seeds, rhizobial inoculum, and AM inoculum were prepared similar to the biostimulation process. The seed of Jack bean (*Canavalia* sp.) were surface sterilized as mention earlier.

Peat moss were used as plant substrate. They were sterile twice by autoclaving at 100°C, 15 psi for 1 hour. Plastic pot used for planting was cleaned by 70% ethylalcohol.

Commercial diesel fuel was mixed with the peat moss at a concentration of 1.05 % (w/w). The treatment consisted of control soil or diesel contaminated soil with only jack bean, and the proper amount of AM spores obtained from previous biostimulation section or 10 g rhizobium or the mixture of both AM and rhizobium amended in soil or diesel contaminated soil with jackbean. The concluded scheme of the experiment was shown in Table 3.3. Complete randomized design (CRD) of 10 pots with 3 replicates of each soil treatment was also used for this experiment. The study was conducted in greenhouse with daily watering and Bell's nutrient solution was weekly added.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Table 3.3 Detailed amendment of each phytoremediation experiment

Experiment	Treatment*	Rhizobium fertilizer (g)	AM (spores)	Jack bean	Diesel oil
11	P'	-	-	✓	-
12	R+P'	10	-	✓	-
13	A50+P'	-	50	✓	-
14	A100+P'	-	100	✓	-
15	R+A50+P'	10	50	✓	-
16	R+A100+P'	10	100	✓	-
17	NP	-	-	-	✓
18	P	-	-	✓	✓
19	R+P	10	-	✓	✓
20	A50+P	-	50	✓	✓
21	A100+P	-	100	✓	✓
22	R+A50+P	10	50	✓	✓
23	R+A100+P	10	100	✓	✓

* P': plant without inoculation, P: plant without inoculation with oil, R+P: 10 g rhizobium with plant, A50+P: 50 spores AM with plant, A100+P: 100 spores AM with plant, R+A50+P: 10 g rhizobium and 50 spores AM with plant, R+A100+P: 10 g rhizobium and 100 spores AM with plant

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

3.5 Analytical procedures

Three months after inoculation, plant growth, biomass, and %AM infection on plant roots were evaluated. Plant growth has been evaluated by height and stem diameter. Biomass was determined by plant dry matter obtained by heating at 40 °C for 12 hr. The appearance of plants was also evaluated to determine the effect of diesel oil on plant growth.

Soil and plant samples were extracted using hexane to separate the remained oil. To assess hydrocarbon removal, 2 g of soil sample was extracted twice with 3 ml of n-hexane in an ultrasonic bath, and the extracts were combined. Plant dry matter was analysed after heating at 40°C for 12 h. Roots were rinsed at least five times with distilled water before drying. Shoots and roots were extracted to determine possible hydrocarbon uptake. They were extracted twice (6 and 3 ml of n-hexane) in an ultrasonic bath. The combined extract was allowed to evaporate to dryness under a fume hood and was concentrated to 2 ml.

The extracts were hydrocarbons analyzed using gas chromatography-mass spectrometry (GC-MS) HP-6890. The GC was equipped with cross-linked 5% phenyl methyl siloxane capillary column, HP-5MS (Figure 3.2). Helium was used as carrier gas. The temperature program was started at 40 °C with the heating rate of 10 °C /min until 300 °C then maintained for 8 min.

Figure 3.2. Agilent Technologies 6890 gas chromatograph.

One-way analysis of variance (ANOVA) using SPSS® software was used to compare the effect of each factor in each treatment by LSD. Differences were considered significant at $p < 0.05$.

CHAPTER IV

RESULTS AND DISCUSSIONS

RESULTS

4.1 AM fungal

The AM spores were isolated from soil and plant roots collected from various places. Single spore of each isolate was tested for the infection and spore productivity on *Sorghum bicolor*. The AM isolate that exhibited the highest number of spores and %AM infection derived from *Acacia mangium* sampling in Nakonratchasima (Table 4.1). This isolate was characterized as *Glomus mosseae* which formed yellow-brown to brown sporocarps. The peridium surrounding these spores was 10-38 µm thick, with robust hyphae mixed with many finer branched hyphae. The whole spores were dark orange-brown or yellow-brown, globose to subglobose or irregular shape which spore diameter sized around 195 µm (Fig. 4.1). The spore walls comprised of three layers (L1, L2 and L3) in which the outer two layers often slough to vary degrees in mature or older spores (especially those in field soils). Characteristics of spore layers are shown below (Bernhard, 2002).

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Table 4.1 The numbers of spore production and %AM infection of each plant species

Plant species	Sampling site	Average number of spore per 100g of soil (n=5)	AM infection (%)
Acacia mangium Willd.	Nakonratchasima	800	42.8
Glycine max (L.) Merr.	Nakonratchasima	400	23.8
Vigna radiate (L.) Wilczek	Nakhonsawan	310	19.5
Sorghum bicolor (L.) Moench	Nakhonsawan	120	15.3
Zea mays L.	Yasothon	350	20.6
Allium ascalonicum L.	Yasothon	590	34.7
Brachiaria ruziziensis R. Germ. & C. M. Evrard	Khonkaen	220	18.4
Morus alba L.	Khonkaen	700	42.5
Tectona grandis L.f.	Phetchaburi	500	28.9
Tamarindus indica L.	Phetchaburi	390	24.1
Parkia speciosa Hassk.	Lopburi	210	16.7
Poinciana pulcherrima L.	Lopburi	330	19.1
Saccharum officinarum L.	Bangkok	430	25.6
Carica papaya L.	Bangkok	650	39.6

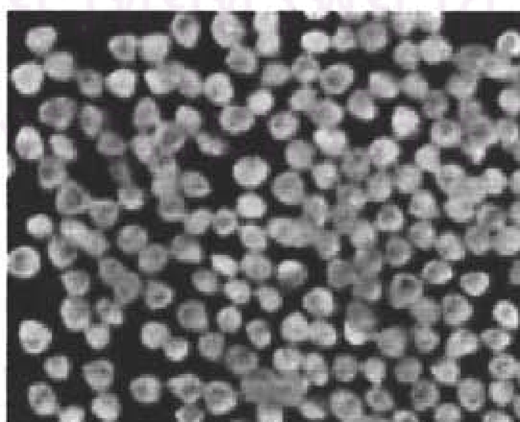


Figure 4.1. *Glomus mosseae* spores

L1: Hyaline, mucilaginous membrane, 1.4-2.5 µm thick, the spores stained pinkish-red in Melzer's reagent, often degrading and then forming a sloughing granular layer, sloughing in mature spores, appearing granular in advanced stages of degradation.

L2: Hyaline, 0.8-1.6 µm thick, very refractile when viewed with differential contrast optics, generally rigid and fracturing into sliver-like fragments observed when this layer separates from L3. It must be attached firmly to the underlying laminae because small irregularly-shaped and shallow pits appear as parts of this layer break away with application of pressure. This layer are not reactive in Melzer's reagent (Morton and Benny, 1990).

L3: A layer consisting of yellow brown to pale orange-brown, sublayers or laminae, 3.2-6.4 µm thick, minute depressions cover the surface with separation of L1 and L2. The subtending hyphae have flared to funnel-shaped (Figure 4.2). Another species often misidentified as *G. mosseae* is *G. caledonium*. However, spore wall structure of *G. caledonium* has four layers, each with distinct phenotypes (Gerdermann and Nicolson, 1963).

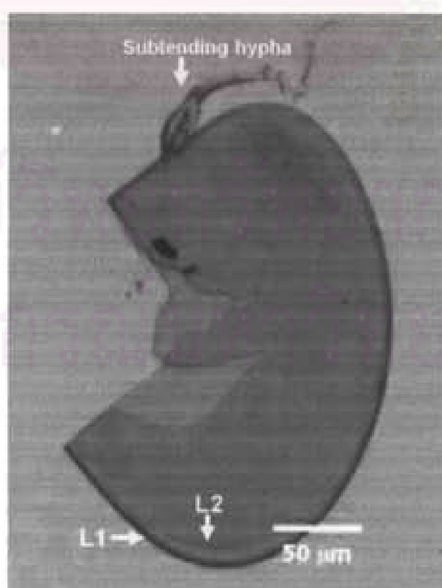
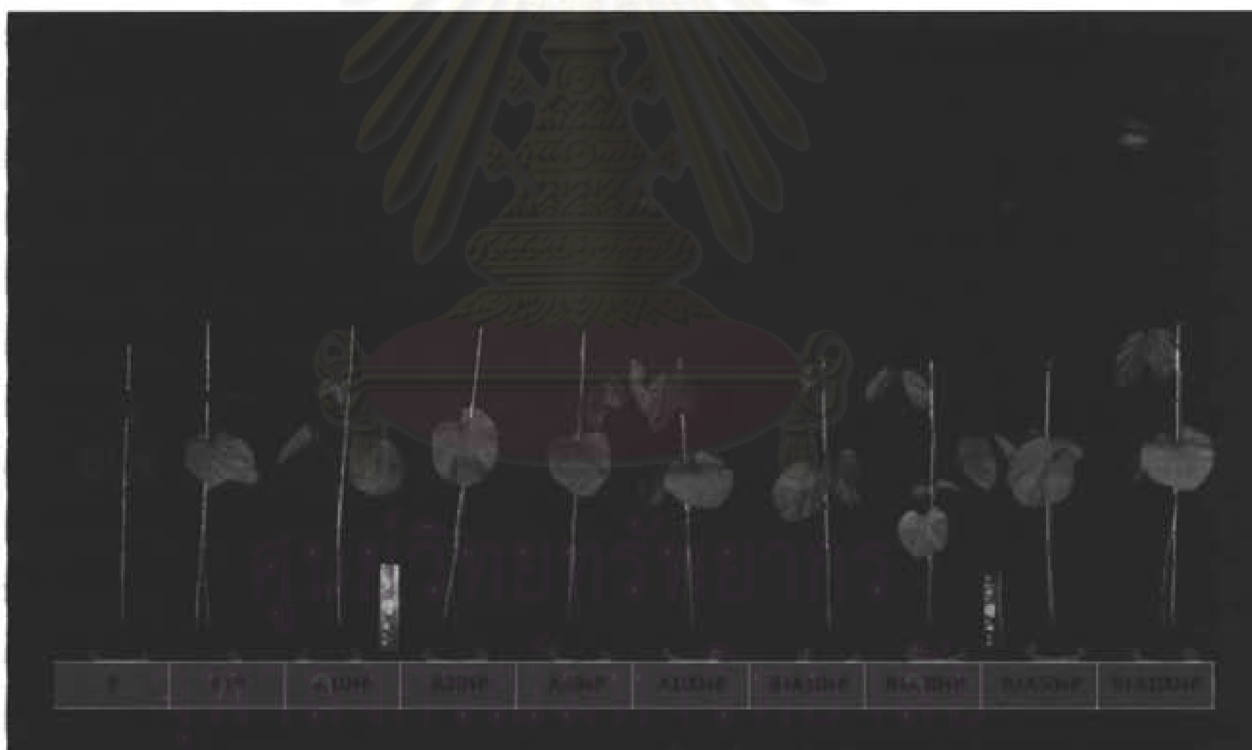


Figure 4.2. An Isolated AM spore from *Acacia mangium* identified into genus *Glomus mosseae*, L: spore wall layer

4.2 Biostimulation

Biostimulation of AM, rhizobium, or combination of AM and rhizobium was determined on Jack bean (*Canavalia* sp.) as plant growth parameter; height and stem diameter and biomass; dry weight of shoot and root. The jack bean growth parameters and biomass after 3 months significantly increased when inoculated with AM and/or rhizobium comparing to control plants without microorganisms (Table 4.2, Figure 4.3). The highest plant growth parameter and biomass were showed in plants inoculated with 100 AM spores and 10 g rhizobium. However, 50 AM spores and 10 g rhizobium could also increase plant growth at the same level as 100 AM spores and 10 g rhizobium.



P: plant without inoculation, R+P: 10 g rhizobium with plant, A+P: AM with plant, R+A+P: 10 g rhizobium and AM with plant

Figure 4.3 Appearances of plant growth amended with AM and/or rhizobium

Typical hypha and vesicles were observed in all AM treatments. The highest AM infection at 72.8% on plant root was observed in plants treated with 10 g rhizobium and 100 spores AM. In addition, the results showed synergistic effects of rhizobium on AM infection in that all treatments of plants inoculated with varying numbers of AM spores alone had lower %AM infection than plants inoculated with both 10 g rhizobium and AM spores. No AM infection was observed in plant root without inoculation of AM spores (Table 4.2).

Table 4.2. Plant growth parameters and biomass of Jack bean after biostimulation

Treatment	Rhizobium fertilizer (g)	AM (spores)	Plant growth		Biomass		AM infection (%)
			Stem height (cm)	Stem diameter (mm)	Shoot (g)	Root (g)	
P	-	-	29.3g	1.72f	1.94e	0.35f	-
R+P	10	-	34.9e	2.15de	2.38d	0.66d	-
A10+P	-	10	33.2f	2.09e	2.25d	0.58e	17.8f
A20+P	-	20	41.4c	2.43c	2.92c	0.71c	28.7e
A50+P	-	50	43.5b	2.87b	3.36b	0.74c	54.2c
R+A10+P	10	10	38.7d	2.28cd	2.74c	0.73c	41.3d
R+A20+P	10	20	44.8ab	2.95ab	3.43b	0.87b	55.6b
R+A50+P	10	50	46.3a	3.08a	3.76a	0.95a	68.4a

P: plant without inoculation, R+P: 10 g rhizobium with plant, A+P: AM with plant, R+A+P: 10g rhizobium and AM with plant

*For each parameter, means followed by the same letter are not significantly different at $p < 0.05$ using LSD.

ศูนย์วิทยทรัพยากร จุฬาลงกรณ์มหาวิทยาลัย

4.3 Effect of microorganisms on enhancing plant growth on contaminated soil

Plant growth, biomass and %AM infection on roots were evaluated in comparing to plants grown in normal soil and diesel contaminated soil with and without rhizobium and AM treatment. All treatments of plants grown in diesel contaminated soil showed less stem height and diameter, biomass, and %AM infection than plants grow in normal soil (Table 4.3). Adding rhizobium and AM in contaminated soil significantly increased growth and biomass of plants compared to plants solely grew in contaminated soil. However, the results showed that growth, biomass, and %AM infection of plants in contaminated soil decreased compared to non-contaminated soil even in combination of AM and rhizobium treatments.

Table 4.3 Comparison of plant growth parameters, biomass, and %AM infection of Jack bean grown on normal soil and diesel contaminated soil.

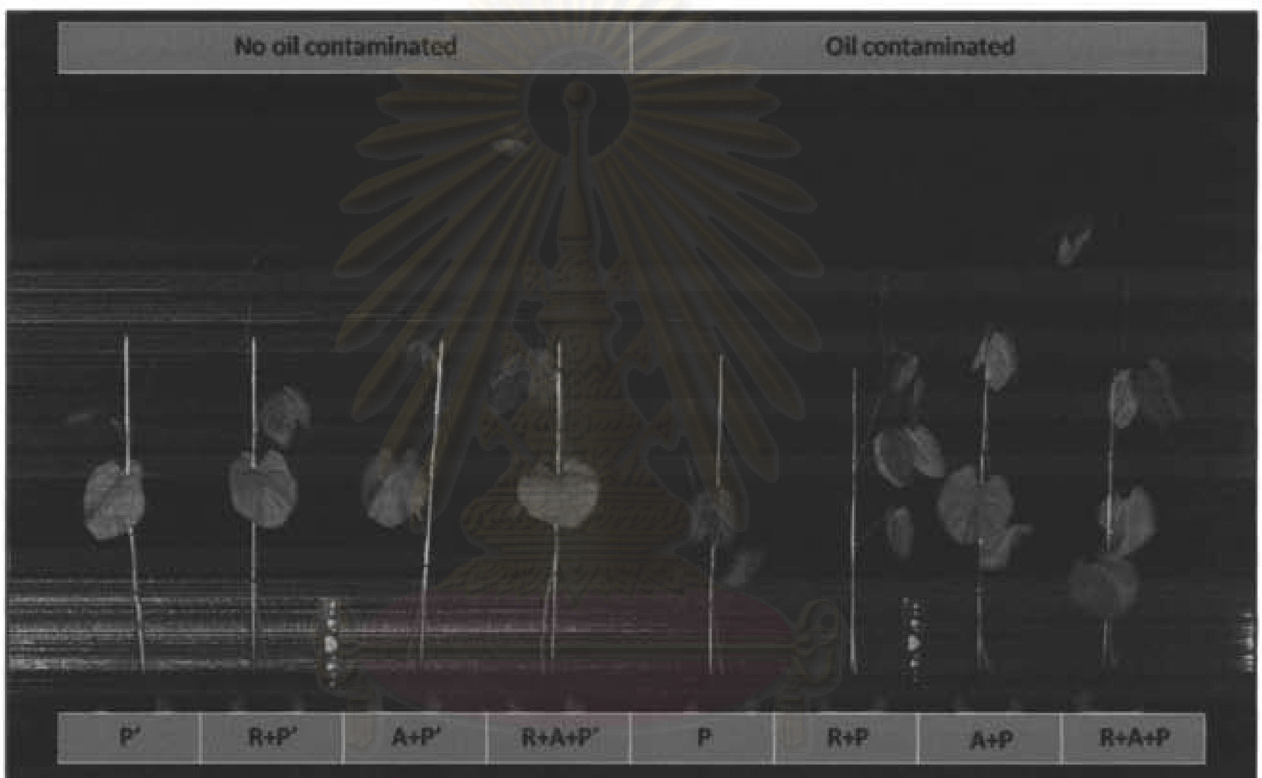
Treatment	Rhizobium fertilizer (g)	AM (spores)	Diesel oil	Plant growth		Biomass		AM infection (%)
				Stem height (cm)	Stem diameter (mm)	Shoot (g)	Root (g)	
P'	-	-	-	29.3e	1.72g	1.94e	0.35f	-
R+P'	10	-	-	34.9d	2.15e	2.38e	0.66c	-
A50+P'	-	50	-	43.5bc	2.87c	3.36c	0.74b	54.2e
A100+P'	-	100	-	43.7bc	2.94abc	3.51b	0.76b	65.7c
R+A50+P'	10	50	-	46.3a	3.08ab	3.76a	0.95a	68.4b
R+A100+P'	10	100	-	46.5a	3.13a	3.82a	0.96a	72.8a
P	-	-	✓	25.6f	0.87h	1.02g	0.13g	-
R+P	10	-	✓	31.2e	1.91f	1.73f	0.45e	-
A50+P	-	50	✓	41.9c	2.45d	2.87d	0.56d	40.3g
A100+P	-	100	✓	42.1c	2.47d	2.9d	0.57d	48.5f
R+A50+P	10	50	✓	45.1ab	2.89bc	3.41bc	0.78b	59.6d
R+A100+P	10	100	✓	45.3ab	2.92bc	3.43bc	0.79b	65.1c

P': plant without inoculation, P: plant without inoculation with oil R+P: 10 g rhizobium with plant, A50+P: 50 spores AM with plant, R+A50+P: 10 g rhizobium and 50 spores AM with plant, A100+P: 100 spores AM with plant, R+A100+P: 10 g rhizobium and 100 spores AM with plant

*For each parameter, means followed by the same letter are not significantly different at $p < 0.05$ using LSD.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

The appearance of plants was also evaluated to determine the effect of diesel oil on plant growth. The plant leaves in contaminated soil exhibited diminutive characteristic compared with plant leaves in non-contaminated soil. The rhizobium and AM inoculation could lessen this effect (Figure 4.4). Phytotoxicity of diesel oil has been reported as a result of reduction in plant growth and appearance (Vouillamoz and Milke, 2001).



P': plant without inoculation, P: plant without inoculation with oil, R+P: 10 g rhizobium with plant, A+P: AM with plant, R+A+P: 10 g rhizobium and AM with plant

Figure 4.4. Appearances of plant growth on normal soil and contaminated soil.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

4.4 Phytoremediation

4.4.1 Analysis of pure diesel oil

The diesel oil was hydrocarbons analyzed using gas chromatography-mass spectrometry (GC-MS). They were performed a qualitative study to identify each separated component by match the MS-spectrum from experiment with the available mass spectrometry-spectrum library. A quantitative study was also performed by calculate an area percent of each separated component as presented in Equation 4.1.

$$\text{Area percent of component X} = \frac{\text{Area of component X}}{\text{Total area of all components}} \times 100 \qquad (4.1)$$

The analysis of the gas chromatograms of pure diesel oil showed 161 hydrocarbon products found in diesel oil. In this experiment, the 19 major hydrocarbon products that exhibited the area percentage higher than 1% were quantitatively analyzed to identify the efficiency of phytoremediation process of each treatment (Figure 4.5).

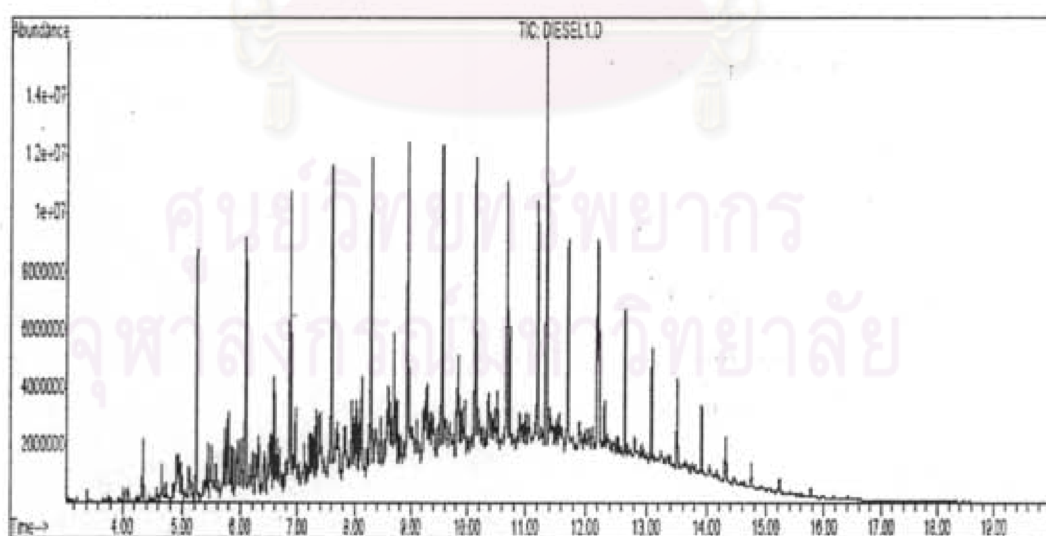


Figure 4.5 Gas chromatograms of pure diesel oil.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Identification of 19 major hydrocarbon products was done by matching the mass spectrum from each separated peak with mass spectrum from library. The hydrocarbon components of diesel oil ranged from short chain hydrocarbon such as nonane, decane, and undecane; branch chain hydrocarbon ranged from dodecane to hexadecanoic acid; more branch chain hydrocarbon consisting of docosane, heneicosane, and 9-octadecenoic acid. (Table 4.4).

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Table 4.4 Major constituents of pure diesel oil.

	Number	Retention time	Chemical name	Chemical structure	%Peak area
Short chain	1	4.342	Nonane		0.31
	2	5.243	Decane		1.41
	3	6.090	Undecane		1.40
Branch chain	4	6.873	Dodecane		2.05
	5	7.606	Tridecane		1.96
	6	8.297	Tetradecane		2.50
	7	8.696	2,10-dimethyl-undecane		1.13
	8	8.944	Pentadecane		2.64
	9	9.015	2-allyl-1-methylnaphthalene		1.02
	10	9.252	2,3,6-trimethyl-naphthalene		1.44
	11	9.554	Hexadecane		2.29
	12	9.835	2,6,10-trimethyl-pentadecane		1.20
	13	10.137	Octadecane		2.18
	14	10.741	2,6,10,14-tetramethyl-hexadecane		1.43
	15	11.211	Nonadecane		1.85
	16	11.351	Hexadecanoic acid		2.65
More branch chain	17	11.712	Docosane		1.90
	18	12.193	Heneicosane		1.47
	19	12.220	9-octadecenoic acid		1.48

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

4.4.2 Analysis of diesel oil extracted from soil

Soil samples of each treatment were extracted using hexane to separate the remained oil. The extracts were hydrocarbons analyzed using gas chromatography-mass spectrometry (GC-MS) comparing between day 0 which was the diesel oil extracted before phytoremediation, and day 90 that represented the diesel oil remained in soil 3 months after inoculation. The analysis showed 19 major hydrocarbons found in diesel oil inwhich significant decrease amounts of hydrocarbons were observed in contaminated soil treated with *Canavalia* sp. amended with rhizobium and/or AM after 3 months (Table 4.5). The results showed that only plants grew on contaminated soil had less effect on remediation, but rhizobium and AM can enhanced diesel removal in phytoremediation. Combination of plant with rhizobium and AM could decrease hydrocarbons in soil better than only plant with rhizobium or plant with AM alone. *Canavalia* sp. stimulated by 10 g rhizobium and 100 spores AM was the most efficient combination to remove diesel contamination. However, 50 and 100 spores AM in soil didn't results in different decrease in hydrocarbons.

From contaminated soil analysis, the linear short chain hydrocarbons, i.e., nonane to undecane, were no longer detected in any treatment after 3 months due to volatization according to the study of Chaineau et al. (2000). More branched or long chain hydrocarbons, i.e., docosane, heneicosane, and 9-octadecenoic acid, were still present in high amounts at even after 3 months, indicating that diesel contaminated was strongly bind to the soil particles (Cunningham et al. 1996) which was dificult to remove by plants.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Table 4.5 Major constituents of diesel oil extracted from soil.

	No.	Retention time	Chemical name	Peak area (%)*							
				Day 0	Day 90						
					NP	P	R+P	A+P	R+A+P	A*+P	R+A*+P
Short chain	1	4.342	Nonane	0.31a	0.00b	0.00b	0.00b	0.00b	0.00b	0.00b	0.00b
	2	5.243	Decane	1.41a	0.00b	0.00b	0.00b	0.00b	0.00b	0.00b	0.00b
	3	6.090	Undecane	1.40a	0.00b	0.00b	0.00b	0.00b	0.00b	0.00b	0.00b
Branch chain	4	6.873	Dodecane	2.05a	1.87b	1.79c	0.71e	0.89d	0.22f	0.85d	0.20f
	5	7.606	Tridecane	1.96a	1.79b	1.73b	0.69d	0.91c	0.25e	0.89c	0.22e
	6	8.297	Tetradecane	2.50a	2.30b	2.21c	0.91e	1.23d	0.39f	1.18d	0.39f
	7	8.696	2,10-dimethyl-undecane	1.13a	1.05b	1.01b	0.42d	0.57c	0.19e	0.55c	0.18e
	8	8.944	Pentadecane	2.64a	2.45b	2.46b	2.25c	2.23c	2.01d	2.20c	1.95d
	9	9.015	2-allyl-1-methylnaphthalene	1.02a	0.96ab	0.96ab	0.88bc	0.88bc	0.81c	0.86c	0.80c
	10	9.252	2,3,6-trimethyl-naphthalene	1.44a	1.37a	1.37a	1.26b	1.25bc	1.16cd	1.23bc	1.13d
	11	9.554	Hexadecane	2.29a	2.18b	2.16b	2.02c	2.00cd	1.86de	1.93cd	1.80e
	12	9.835	2,6,10-trimethyl-pentadecane	1.20a	1.15ab	1.15ab	1.09bc	1.06bcd	1.00cd	1.04cd	0.97d
	13	10.137	Octadecane	2.18a	2.08b	2.07b	1.99bc	1.94c	1.81de	1.90cd	1.78e
	14	10.741	2,6,10,14-tetramethyl-hexadecane	1.43a	1.38ab	1.37ab	1.31bc	1.28cd	1.20de	1.27cd	1.16f
	15	11.211	Nonadecane	1.85a	1.78ab	1.77abc	1.71bcd	1.67cd	1.55ef	1.6de	1.51f
	16	11.351	Hexadecanoic acid	2.65a	2.56ab	2.53bc	2.42d	2.44cd	2.24e	2.36d	2.19e
More branch chain	17	11.712	Docosane	1.90a	1.87ab	1.83abc	1.76c	1.79bc	1.62d	1.76c	1.60d
	18	12.193	Heneicosane	1.47a	1.44ab	1.42abc	1.36c	1.38bc	1.27d	1.36c	1.26d
	19	12.220	9-octadecenoic acid	1.48a	1.46ab	1.43abc	1.36cd	1.39bc	1.29de	1.38c	1.29e

day 0: peak area of contaminated soil extracted at day 0, day 90: peak area of contaminated soil extracted at day 90, NP: no plant , P: plant without inoculation, R+P: 10 g rhizobium with plant, A+P: 50 spores AM with plant, R+A+P: 10 g rhizobium and 50 spores AM with plant, A+P: 100 spores AM with plant, R+A*+P: 10 g rhizobium and 100 spores AM with plant

**For each peak area, means followed by the same letter are not significantly different at $p < 0.05$ using LSD

4.4.3 Analysis of diesel oil extracted from shoot and root

In order to determine the ability of *Canavalia* sp. to remediate diesel contaminated soil, hydrocarbon concentrations in shoot and root tissues were also analyzed. Shoot and root tissue samples of each treatment were extracted using hexane to separate the diesel oil from plant cells. The extracts were hydrocarbons analyzed using gas chromatography-mass spectrometry (GC-MS) at the same procedure as diesel oil extraction in soil. The results of percentage of peak area of 19 major components exist in shoot tissues were shown in Table 4.6. The hydrocarbon contaminants in roots was also analyzed but it was too low compare with components in shoot which were lower than 1% (data not shown).

From shoot analysis, the short chain hydrocarbons, i.e., nonane to undecane, were not detectable in any treatment after three months as same as the result from soil analysis. Branch chain hydrocarbons; dodecane, tridecane, tetradecane and 2,10-dimethyl-undecane, were detected in higher amounts in shoot than in phytoremediated soil treated with microorganisms indicating the ability of plants to extract these types of hydrocarbons from soil. In contrast, the high molecular weight and more branch chain hydrocarbons were left in soil in higher amounts than absorbed in to plants which may resulted from the strong binding of the hydrocarbons to the soil particles (Table 4.5 and 4.6).

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Table 4.6 Major constituents of diesel oil extracted from shoot.

	No.	Retention time	Chemical name	Peak area (%)*					
				Day 90					
				P	R+P	A+P	R+A+P	A*+P	R+A*+P
Short chain	1	4.342	Nonane	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
	2	5.243	Decane	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
	3	6.090	Undecane	0.00a	0.00a	0.00a	0.00a	0.00a	0.00a
Branch chain	4	6.873	Dodecane	0.32d	1.16b	0.98c	1.65a	1.02c	1.68a
	5	7.606	Tridecane	0.29d	1.09b	0.87c	1.54a	0.89c	1.58a
	6	8.297	Tetradecane	0.36d	1.39b	1.04c	1.89a	1.09c	1.89a
	7	8.696	2,10-dimethyl-undecane	0.16d	0.61b	0.47c	0.84a	0.49c	0.86a
	8	8.944	Pentadecane	0.24d	0.27cd	0.28cd	0.50b	0.31c	0.56a
	9	9.015	2-allyl-1-methylnaphthalene	0.09c	0.10bc	0.10bc	0.17a	0.12b	0.18a
	10	9.252	2,3,6-trimethyl-naphthalene	0.12b	0.12b	0.13b	0.21a	0.15b	0.24a
	11	9.554	Hexadecane	0.15d	0.18d	0.19d	0.31b	0.26c	0.37a
	12	9.835	2,6,10-trimethyl-pentadecane	0.07d	0.07d	0.10cd	0.16b	0.12c	0.19a
	13	10.137	Octadecane	0.12de	0.11e	0.14d	0.28b	0.19c	0.32a
	14	10.741	2,6,10,14-tetramethyl-hexadecane	0.07d	0.08d	0.10cd	0.19b	0.11c	0.22a
	15	11.211	Nonadecane	0.06e	0.08e	0.11d	0.23b	0.15c	0.27a
	16	11.351	Hexadecanoic acid	0.08e	0.14d	0.11de	0.30b	0.19c	0.36a
More branch chain	17	11.712	Docosane	0.05c	0.09b	0.05c	0.22a	0.09b	0.24a
	18	12.193	Heneicosane	0.02d	0.07b	0.04c	0.15a	0.07b	0.16a
	19	12.220	9-octadecenoic acid	0.02d	0.07b	0.04c	0.14a	0.05c	0.15a

day 90: peak area of contaminated soil extracted at day 90, P: plant without inoculation, R+P: 10 g rhizobium with plant, A+P: 50 spores AM with plant, R+A+P: 10 g rhizobium and 50 spores AM with plant, A+P: 100 spores AM with plant, R+A*+P: 10 g rhizobium and 100 spores AM with plant

**For each peak area, means followed by the same letter are not significantly different at p < 0.05 using LSD

DISCUSSIONS

AM and rhizobium can stimulate Jack bean growth due to the synergistic interaction between AM and rhizobium species and other rhizobacteria. It has been reported in that AM fungi improved nodulation due to enhanced P-uptake and supplied trace elements by the plants (Albrecht et. al. 1999; Khan, 2006). Plants inoculated with rhizobium also showed a greater N content than control plants. Thus increases in P content of plants inoculated with *Rhizobium* sp. and *Glomus coronatum* can be also associated with higher N contents due to increase of N fixation (Requena et al. 1997). Co-inoculation of rhizobium and mycorrhiza was reported to produce growth and nutrient uptake, together with a noticeable increase in mycorrhizal root colonization (Barea et al. 1997). Jack bean co-inoculated with 10 g rhizobium and 100 spores AM in this experiment also showed increase in %AM infection which may resulted from high number of plant roots and increase in mycorrhizal colonization.

Diesel contaminated soil decreased plant growth and biomass of Jack bean. This pollutants generally have strong negative effects on the plant community both on direct and indirect effects. The direct effects of hydrocarbons are their toxicity and interfere physical structure of soil that lead to decrease capacity to store moisture and air. The indirect effects are their interactions with the abiotic and microbial components of soil that bring about the microbial production of phytotoxic compounds (Wang and Bartha, 1994). Moreover, the high concentrations of contaminants tend to inhibit plant growth, due to oxidative stress (Chaudhry et al. 2005). Contaminated soils also tend to lack of microbial diversity. There are several potential reasons for lack of efficient microbial remediation. These include the inability of introduced microbes to compete with existing microflora in soil, the inability of microbes to grow to sufficient depths to reach sub-surface contaminants, insufficient nutrients in contaminated soils to support microbial growth, low bioavailability of contaminants, preferential utilization of other carbon compounds than the contaminant interest, and the presence of toxicants at the site that inhibit microbial growth (Kuiper et al. 2004).

Adding of rhizobium and AM in phytoremediation of diesel oil with Jack bean showed the ability to reduce the phytotoxicity of diesel in soil. The reduction of phytotoxicity and stimulating the degradation of other non- phytotoxic contaminants were involved by activities of many microbes (Siciliano and Germida, 1998). In fact, Walton et al. (1994) have hypothesized that the defenses of plants to contaminants may be supplemented through the external degradation of contaminants by microorganisms in the rhizosphere. Plants and microbs have co-evolved a mutually-beneficial strategy for dealing with phytotoxicity, where microorganisms benefit from the plant exudates while the plants benefit from the ability of microorganisms to break down toxic chemicals.

Adequate soil nutrients are required to support the growth of plants and their associated microorganisms. This may be especially true during phytoremediation efforts, when the plant/microbe community is already under stress from the contaminant. Xu and Johnson (1997) have shown that petroleum hydrocarbons can significantly reduce the availability of plant nutrients in soil. Low nutrient availability results from the fact that petroleum hydrocarbons have high carbon contents, but are poor suppliers of nitrogen and phosphorus. As soil microorganisms degrade the hydrocarbons, they use up or immobilize available nutrients (i.e., nitrogen and phosphorus) creating nutrient deficiencies in contaminated soil. Biederbeck et al. (1993) found that, following initial applications of an oily waste sludge to sandy soil, the soil had very low nitrate levels due to immobilization of nitrogen by rapidly growing populations of oil degrading bacteria as well as suppression of nitrogen-fixing bacteria. Two years following oil application, however, sludge treated plots contained more nitrate than untreated controls, presumably due to the gradual remineralization of the previously immobilized nitrogen. The trend was similar for phosphorus, which was initially low following incorporation due to immobilization by an expanding microbial biomass, but became more available one year later. Petroleum hydrocarbons also may limit the accessibility of nutrients to plants and microorganisms by reducing the availability of water in which the nutrients are dissolved (Schwendinger, 1968). Nutrient deficiencies in soil caused by petroleum hydrocarbons may be offset by the application of fertilizer or *green manure* to the soil.

In addition, the presense of rhizobium or AM in soil could increase soil nutrients and influence bioavailability of *Canavalia* sp.. Inoculation of rhizobium and AM into contaminated soil could alleviate the effect of diesel on plant growth. The benefit of AM spores and rhizobium amendment is that organic materials, nutrients, and oxygen are added to soil via plant and microbial metabolic processes. This improves the overall quality and texture of soil at remediated sites (Gerhardt et al. 2009).

There was evidence that some plants can provide the movement of hydrophobic organics such as PAHs from soil into the rhizosphere (Liste and Alexander, 2000). Mobilization of PAHs may be accelerated by the release of organic acids from roots, which putatively increase PAH solubility and bioavailability.

Models using the octanol-water partition coefficient have been developed and tested to varying degrees for the uptake of organic chemicals by plants (Paterson et al. 1994; Trapp et al. 1990). The octanol-water partition coefficient (K_{ow}, often expressed as $\log K_{ow}$) is a measure of a chemical's affinity for water versus lipids or fats. In general, chemicals that are highly water soluble (i.e.*hydrophilic* compounds with a $\log K_{ow} < 0.5$) are not sufficiently sorbed to roots or actively Transported through plant membranes (Schnoor et al. 1995). Hydrophobic chemicals ($\log K_{ow} > 3.0$) are not easily transported within the plant because they are strongly bound to and may not pass beyond the root's surface due to the high proportion of lipids present at the surface (Siciliano and Germida, 1998). Two exceptions are the uptake of polychlorinated dibenzo-*p*dioxins and dibenzofurans ($\log K_{ow} > 6$) by the roots of zucchini and pumpkin (Hulster et al. 1994).

Moderately *hydrophobic* organic chemicals ($\log K_{ow} = 0.5$ to 3.0) are effectively taken up by plants; these chemicals include most BTEX, chlorinated solvents, and short chain aliphatic chemicals (Schnoor et al. 1995; Siciliano and Germida, 1998). In addition to the octanol-water partition coefficient, the size and molecular weight of an organic contaminant may play a role in the ability of a plant to take up the contaminant. Anderson et al., 1993 reported that plant root uptake usually favors small, low molecular weight polar compounds, whereas large, high molecular weight compounds tend to be excluded from the root. Environmental conditions and plant characteristics also may affect uptake by roots (Anderson et al. 1993).

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Phytoremediation of diesel contaminated soil was successfully done by Jack bean and combination of AM and rhizobium. The major hydrocarbons found in diesel oil were decrease in contaminated soil and were accumulated in shoot of Jack bean.

Various studies have documented the accumulation of petroleum hydrocarbons in plants as well as the adsorption of these compounds onto the plants tissue. Researchers have identified that the lipid content of the plant may influence the degree of accumulation of petroleum hydrocarbons. Attempts have been made to model the uptake and accumulation of petroleum hydrocarbons in plants based on the chemical characteristics of the various hydrocarbons, particularly the compound's affinity for lipids using radioactive hydrocarbons [^{14}C]. The [^{14}C]benzene in soils planted with alfalfa and [^{14}C]anthracene in soil planted with soybean were recovered in plant tissues and shoots (Ferro et al. 1997 and Edwards et al. 1982). Edwards (1988) also investigated the uptake and translocation of [^{14}C]anthracene and [^{14}C]benz[*a*]anthracene in bush bean, using plants grown in a nutrient solution. Results indicated ^{14}C dose was taken up into the shoots. This indicates that anthracene (the smaller, more water-soluble of the two PAHs), together with its metabolites, is more readily translocated from the roots and assimilated within the plant tissues.

Hydrocarbons uptake strategy is based on hydrocarbons uptake into roots, preferential storage of hydrocarbons in root vacuoles and restricted translocation into shoots. Hyperaccumulator, in contrast, take up more hydrocarbons, store a lower proportion of them in root vacuoles, and export higher amounts to shoots. The pathways of redial root transport of hydrocarbons into the vascular cylinder are a further issue in hyperaccumulation research that apoplastic transport of hydrocarbons to the xylem is required for sustain in hydrocarbons transport to shoots (Lasat et al. 2000).

The presense of hydrocarbons in shoots of Jack bean treated with rhizobium and AM indicated that phytoaccumulation was occurred and played a role in diesel contaminated removed in soil. With in three months of this phytoremediation could eliminate some level of diesel in soil. Thus for successfully remediate diesel contaminated soil by Jack bean co-inoculated with 10 g rhizobium and 100 spores AM, Plants should be left in the field in longer period of times. The little amount of

hydrocarbons were detected in Jack bean roots indicating that harvest of Jack bean for phytoremediation will be very effective.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

CHAPTER V

CONCLUSIONS

In order to obtain the effective solution in removal of oil contamination, the effects of rhizobium and mycorrhiza enhanced *Canavalia* sp. in phytoremediation of diesel oil contaminated in soil. This experiments were investigated through plant growth parameter and % of remaining hydrocarbon species. The following is a summary derived from this work.

1. Each rhizobium or AM can enhanced bioavailibility of *Canavalia* sp. resulting in higher plant growth parameter compared with *Canavalia* sp. alone. This effectiveness was synergistic in treatment with both rhizobium and AM. The best growth parameter was obtain by using 10 g rhizobium with 100 spores of AM that significantly enhanced stem height, stem diameter, shoot dry weight, root dry weight, and %AM infection on root.
2. Diesel oil mainly consists of hydrocarbon that divided into two groups, short chain or low molecular weight hydrocarbon which can be detoxified by volatization, another contains long branched chain or high molecular weight hydrocarbon that has been slightly dissipated by planted with *Canavalia* sp.
3. Inoculation of rhizobium or arbuscular mycorrhiza enhanced bioavailibility of *Canavalia* sp. and also significantly decrease hydrocarbons in soil which hardly eradicate via volatization. Phytoremediation outcome was greater when combination of both rhizobium and arbuscular mycorrhiza symbiosis the remained hydrocarbons in soil was significant decrease.
4. Hydrocarbon concentration in shoot and root tissues showed linear short chain hydrocarbons which were found in plant shoot but not exist in root. This phenomenon proved that the phytoaccumulation was exhibited and played important role in diesel contaminated removal in soil. This outcome was harshly reduced in long chain hydrocarbons that might be due to the strong binding effect of contamination with soil particles.

5. Comparing between phytoremediation treatment of 10 g rhizobium with 50 and 100 spores AM resulted that 100 spores AM slightly indicated more diesel removal efficiency over 50 spores. Then *Canavalia* sp. stimulation using 100 spores AM with 10 g rhizobium expressed the most optimum solutions to remediate diesel oil contamination in soil for this experiment.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

REFERENCES

Abd-Alla, M.H., Omar, S.A., and Karanxha, S. The impact of pesticides on arbuscular mycorrhizal and nitrogen-fixing symbioses in legumes. Applied Soil Ecology Vol. 14 (2000) : 191–200.

Albrecht, C., Geurts, R., Lapeyrie, F. and Bisseling, T. Endomycorrhizae and rhizobial Nod factors both require SYM8 to induce the expression of the early nodulin genes *PsENOD5* and *PsENOD12A*. Plant J Vol. 15(1998) : 605–614.

Albrecht, C., Geurts, R., and Bisseling, T. Legume nodulation and mycorrhizae formation, two extremes in host specificity meet. The EMBO Journal Vol. 18, No.2 (1999) : 281-288.

Anderson, T.A., Guthrie, E.A., and Walton, B.T. Bioremediation in the rhizosphere. Environmental Science and Technology Vol. 27, No.13 (1993) : 2630-2636.

Anderson, T.A., Kruger, E.L., and Coats, J.R. Enhanced degradation of a mixture of three herbicides in the rhizosphere of a herbicide-tolerant plant. Chemosphere Vol. 28 (1994) : 1551–1557.

Andrade, G., Mihara, K.L., Linderman, R.G., and Bethlenfalvay, G.J. Bacteria from the rhizosphere and hyphorhizosphere soils of different arbuscular mycorrhizal fungi. Plant and Soil Vol. 192, No. 1 (1997) : 71-79.

Aprill, W., and Sims, R.C. Evaluation of the use of prairie grasses for stimulating polycyclic aromatic hydrcarbon treatment in soil. Chemosphere Vol.20, No.1-2 (1990) : 253-265.

Ardourel, M., Demont, N., Debelle, F., Maillet, F., DeBilly, F., Prome, J.C., Denarie, J., and Truchet, G. Rhizobium meliloti lipooligosaccharide nodulation factors: different structural requirements for bacterial entry into target root hair cells and induction of plant symbiotic developmental responses. Plant Cell Vol. 6 (1994) : 1357–1374.

Armishaw, R., Bardos, P., Dunn, R., Hill, J., Pearl, M., Rampling, T., and Wood, P. Review of Innovative contaminated Soil Clean-Up Processes. Report LR 819 (MR). Warren Spring Laboratory : Stevenage, UK, SG1 2BX, UK. 1991.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Atkinson, D., Berta, G., and Hooker, J.E. Impact of Mycorrhizal Colonization on Root Architecture, Root Longevity, and the Formation of Growth Regulators. In: Gininazzi, S., Scheupp, H. (Eds.), Impact of Arbuscular Mycorrhizas on Sustainable Agriculture and Natural Ecosystems, 89-99. Basel : Birkhauser Verlag, 1994.

Atlas, R. M., and Bartha, R., Microbial Ecology: Fundamentals and Applications. Benjamin/Cummings Publishing Company, Inc. : Don Mills, ON., 1998.

Balestrini, R., Berta, G., and Bonfante, P. The plant nucleus in mycorrhizal roots: positional and structural modifications. Biology of the Cell Vol. 75 (1992) : 235-243.

Banuelos, G.S., Ajwa, H.A., Mackey, B., Wu, L.L., Cook, C., Akohoue, S., and Zambrzuski, S. Evaluation of different plant species used for phytoremediation of high soil selenium. Journal of Environmental Quality Vol.26 (1997) : 639-646.

Barea, J.M., Azon-Aguilar, C., and Azcon, R. Interactions between Mycorrhizal Fungi and Rhizosphere Microorganisms within the Context of Sustainable Soil-plant System. In: Gange, A.C., Brown, V.K. (Eds.), Multrophic Interactions in Terrestrial Ecosystems, 65-77. Oxford : Blackwell, 1997.

Bernhard, K. Studies on arbuscular mycorrhiza (AM) in the Alentejo (Portugal) using pea mutants Resistant to AM fungi as a control tool for field conditions. Ph.D. Thesis Department of Soil Biology and Plant Nutrition Faculty of Ecological Agricultural Sciences University of Kassel, 2002.

Berti, W.R., and Cunningham, S.D. Phytostabilization of metals. In: Raskin I, Ensley B (eds) Phytoremediation of toxic metals: Using plants to clean up the environment. Wiley Interscience, New York, (2000) : 71-88.

Biederbeck, V.O., St.Jacques, R.M., Curtin, D., Geissler, H.J., Selles, F., and Waddington, J. Use of Heavy Oil Waste Sludge for Protection and Improvement of Sandy Soil. Agriculture and Agri-Food Canada, SK, and Environment, Canada, 1993.

Biermann, B., and Linderman, R.G. Use of vesicular-arbuscular mycorrhizal roots, intraradical vesicles and extraradical vesicles as inoculum. New Phytologist Vol. 95 (1983) : 97-105.

Bossert, I. and Bartha, R. The fate of petroleum in soil ecosystems. Petroleum Microbiology, 435-473. R.M. Atlas, New York. : MacMillan, 1984.

Cardenas, L., Dominquez, J., Quito, C., Lopez-Lara, I.M., Lugtenberg, B.J.J., Spaink, H.P., Rademaker, G.H., Haverkamp, J., and Thomas- Oates, J.E. Isolation, chemical structure and biological activity of the lipo-chitin oligosaccharide nodulation from Rhizobium etli. Plant Molecular Biology Vol. 29 (1995) : 453–464.

Campbell, R. Plant Microbiology. Baltimore, MD. : Edward Arnold, 1985.

Carlson, R.W., Price, N.P.J., and Stacey, G. The biosynthesis of rhizobial lipo-oligosaccharide nodulation signal molecules. Molecular Plant-Microbe Interactions Vol. 7 (1994) : 684–695.

Cerniglia, C.E., Kelly, D.W., Freeman, J.P., and Miller, D.W. Microbial metabolism of pyrene. Chemico-Biological Interactions Vol. 57 (1986) : 203-216.

Chaineau, C.H., Morel, J.L., and Oudot, J. Phytotoxicity and plant uptake of fuel oil hydrocarbons. Journal of Environmental Quality Vol. 29 (1997) : 1478 – 1483.

Chaineau, C.H., Morel, J.L., and Oudot, J. Biodegradation of fuel oil hydrocarbons in the rhizosphere of maize. Journal of Environmental Quality Vol. 29 (2000) : 569–578.

Chaudhry, Q., Blom-Zandstra, M., Gupta, S., and Joner, E.J. Utillizing the synergy between plants and rhizosphere microorganisms to enhance breakdown of organic pollutants in the environment. Environmental Science & Pollution Research Vol. 12 (2005) : 34-48.

Chaudhry, T.M., and Khan, A.G. Plants Growing on Abandoned Mine Site and Their Root Symbionts. In: Gorban, G.R., Lepp, N. (Eds.), Proceedings 7th International Conference on the Biogeochemistry of Trace Elements, 134-135. Uppsala, Sweden : Swedish University of Agricultural Sciences, 2003.

Chin-A-Woeng, T.F.C., Bloemberg, G.V., and Lugtenberg, B.J.J. Phenazines and their role in biocontrol by Pseudomonas bacteria. New Phytologist Vol. 157, No. 3 (2003) : 503-523.

Cunningham, S.D., Anderson, T.A., Schwab, A.P., and Hsu, F.C. Phytoremediation of soils contaminated with organic pollutants. Advances in Agronomy Vol. 56 (1996) : 55-114.

Cunningham, S.D., and Berti, W.R. Remediation of contaminated soils with green plants: An overview. In Vitro Cellular & Developmental Biology Plant Vol. 4 (1993) : 207-212.

Damaj, M., and Ahmad, D. Biodegradation of polychlorinated biphenyls by rhizobia: a novel finding. Biochemical and Biophysical Research Communications Vol. 218 (1996) : 908–915.

Daniels, B.A., and Skipper, H.D. Methods and principles of mycorrhizal research. St. Paul Minnesota : The American Phytopathological Society, 1979.

Diaz, C.L., Spaink, H.P., Wijffelman, C.A., and Kijne, J.W. Genomic requirements of Rhizobium for nodulation of white clover hairy roots transformed with the pea lectin gene. Molecular Plant-Microbe Interactions Vol. 8 (1995) : 348–356.

Donnelly, P.K., Hegde, R.S., and Fletcher, J.S. Growth of PCB-degrading bacteria on compounds from photosynthetic plants. Chemosphere Vol. 28, No. 5 (1994) : 981-988.

Durmishidze, S.V. Metabolism of certain air-polluting organic compounds in plants (review). Applied Biochemistry and Microbiology Vol. 13, No.6 (1977) : 646-653.

Edwards, N.T. Assimilation and metabolism of polycyclic aromatic hydrocarbons by vegetation -an apporach to this controversial issue and suggestions for future research. Polycyclic Aromatic Hydrocarbons: A Decade of Progress - 10th International Symposium, Columus, OH : Battelle Press, 1988.

Edwards, N.T., Ross-Todd, B.M., and Garver, E.G. Uptake and metabolism of ^{14}C anthracene by soybean (Glycine max). Environmental and Experimental Botany Vol. 22, No.3 (1982) : 349-357.

Ehrhardt, D.W., Wais, R., and Long, S.R. Calcium spiking in plant root hairs responding to Rhizobium nodulation signals. Cell Vol. 85 (1996) : 673–681.

Erickson, L.E., Davis, L.C., and Muralidharan, N. Bioenergetics and bioremediation of contaminated soil. Thermochimica Acta Vol. 250 (1995) : 353-358.

Eweis, J.B., Ergas, S.J., Chang, D.P.Y., and Schroeder, E.D. Bioremediation Principles. Toronto : McGraw-Hill, Inc., 1998.

Ezawa, T., Smith, S.E., and Smith, F.A. P metabolism and transport in AM fungi. Plant Soil Vol. 244 (2002) : 221 – 230.

Faucher, C., Camut, S., Denarie, J., and Truchet, G. The nodH and nodQ host range genes of Rhozbium meliloti behave as avirulence genes in *R.leguminosarum* bv. viciae and determine change in the production of plant-specific extracellular signals. Molecular Plant Microbe Interactaction Vol. 6 (1989) : 291–300.

Felle, H.H., Kondorosi, E., Kondorosi, A., and Schultze, M. Rapid alkalinization in alfalfa root hairs in response to rhizobial lipochitooligosaccharide signals. The Plant Journal Vol. 10 (1996) : 295–301.

Felle, H.H., Kondorosi, E., Kobdorosi, A., and Schultze, M. The role of ion fluxes in Nod factor signalling in Medicago sativa. The Plant Journal Vol. 13 (1998) : 455–464.

Ferro, A.M., Kennedy, J., Doucette, W., Nelson, S., Jauregui, G., McFarland, B., and Bugbee, B. Fate of benzene in soils planted with alfalfa: uptake, volatilization, and degradation. Phytoremediation of Soil and Water Contaminants, 223-237. Washington, D.C. : American Chemical Society, 1997.

Fox, T.R., Comerford, N. B., and McFee, W.W. Kinetics of phosphorus release from spores: Effects of oxalate and formate. Soil Science Society of America Journal Vol. 54 (1990) : 1441-1447.

Flathman, P.E., and Lanza, G.R. Phytoremediation: current views on an emerging green technology. Journal of Soil Contamination Vol. 7, No.4 (1998) : 415 – 433.

Freiberg, C., Fellay, R., Bairoch, A., Broughton, W.J., Rosenthal, A., and Perret, X. Molecular basis of symbiosis between Rhizobium and legumes. Nature Vol. 387 (1997) : 394–401.

Garbaye, J., Churin, J.L., and Duponnois, R. Effects of substrate disinfection, fungicide treatments and mycorrhization helper bacteria on ectomycorrhizal formation of pedunculate oak (Quercus robur) inoculated with Laccaria laccata in two bare-root nurseries. Biology and Fertility of Soils Vol. 13, No.1 (1992) : 55-57.

Garbaye, J. Helper bacteria: a new dimension to the mycorrhizal symbiosis. New Phytologist Vol. 128, No. 2 (1994) : 197-210.

Gazey, C., Abbott, L.K., and Robson, A.D. The rate of development of mycorrhizas affects the onset of sporulation and production of external hyphae by two species of *Acaulospora*. Mycological Research Vol. 96 (1992) : 643–650.

Gerdemann, J.W., and Nicolson, T.H. Spores of mycorrhizal Endogone species extracted from soil by wet-sieving and decanting. Transactions of the British Mycological Society Vol. 46 (1963) : 235-244.

Gerhardt, K.E., Huang, X.D., Glick, B.R., and Greenberg, B.M. Phytoremediation and rhizoremediation of organic soil contaminants: Potential and challenges. Plant Science Vol. 176 (2009) : 20-30.

Geurts, R., Heidstra, R., Hadri, A.E., Downie, J.A., Franssen, H., Van Kammen, A., and Bisseling, T. Sym2 of Pisum sativum is involved in a Nodulation factor perception mechanism that controls the infection process in the epidermis. Plant Physiology Vol. 115 (1997) : 351–359.

Gianinazzi-Pearson, V. Plant cell responses to arbuscular mycorrhizal fungi: getting to the roots of the symbiosis. Plant Cell Vol. 8 (1996) : 1871–1883.

Gibson, D.T., and Subramanian, V. Chapter 7 Microbial degradation of aromatic hydrocarbons. Microbial Degradation of Organic Compounds, New York : D.T. Gibson. Marcel Dekker, Inc., 1984.

Giovannetti, M., Avio, L., Sbrana, C., and Citernesi, A.S. Factors affecting appressorium development in the vesicular–arbuscular mycorrhizal fungus Glomus mosseae (Nicol. & Gerd.). New Phytologist Vol. 123 (1993) : 115–122.

Giovannetti, M., and Gianinazzi-Pearson, V. Biodiversity in arbuscular mycorrhizal fungi. Mycological Research Vol. 98 (1994) : 705-715.

Glick, B.R. The enhancement of plant growth by free-living bacteria. Canadian Journal of Microbiology Vol. 41 (1995) : 109-117.

Gudin, C., and Syratt, W.J. Biological aspects of land rehabilitation following hydrocarbon contamination. Environmental Pollution Vol. 8 (1975) : 107-112.

Gunther, T., Dornberger, U., and Fritsche, W. Effects of ryegrass on biodegradation of hydrocarbons in soil. Chemosphere Vol. 33, No. 2 (1996) : 203-215.

Harrison, M.J. Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis. Annual Review of Plant Physiology and Plant Molecular Biology Vol. 50 (1999) : 361–389.

Hegde, R.S., and Fletcher, J.S. Influence of plant growth stage and season on the release of root phenolics by mulberry as related to development of phytoremediation technology. Chemosphere Vol.32, No.13 (1996) : 2471-2479.

Heidstra, R., Geurts, R., Franssen, H., Spaink, H.P., Van Kammen, A., and Bisseling, T. Root hair deformation activity of nodulation factors and their fate on Vicia sativa. Plant Physiology Vol. 105 (1994) : 787–797.

Heijden Van der, M.G.A., Klironomos, J.N., Ursic, M., Moutoglis, P., Streitwolf-Engel, R., Boller, T., Wiemken, T. A., and Sanders, I.R. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. Nature Vol. 396 (1998) : 69 – 72.

Houghton, J.E., and Shanley, M.S. Biological Degradation and Bioremediation of Toxic Chemicals. Portland, Oregon. : G. R. Chaudhry Dioscorides Press, 1994.

Hulster, A., Muller, J. F., and Marschner, H. 1994. Soil-plant transfer of polychorinated dibenzo-pdioxins and dibenzofurans to vegetables of the cucumber family (*Cucurbitaceae*). Environmental Science and Technology. Vol. 28 : 1110-1115.

INVAM/Internationl cultural collection of (Vesilcular) Arbuscular Mycorrhizal fungi [Online]. Availble: http://www. invam.caf.wvu.edu/,1997

Jamal, A., Ayub, N., Usman, M., and Khan, A. Arbuscular Mycorrhizal Fungi Enhance Zinc and Nickel Uptake from Contaminated Soil by Soybean and Lentil. International Journal of Phytoremediation Vol. 4 (2002) : 205-221.

Johnson, D.L., Jones, K.C., Langdon, C.J., Piearce, T.G., and Semple, K.T. Temporal changes in earthworm availability and extractability of polycyclic aromatic hydrocarbons in soil. Soil Biology and Biochemistry Vol. 34 (2002) : 1363–1370.

Joner, E.J., Johansen, A., Loibner, A.P., Dela Cruz, M.A., Szolar, O.H.J., Portal, J.M., and Leyval, C. Rhizosphere effects on microbial community structure and dissipation and toxicity of polycyclic aromatic hydrocarbons (PAHs) in spiked soil, Environmental Science & Technology Vol. 35 (2001) : 2773–2777.

Jones, R., Sun, W., Tang, C.S.,and Robert, F.M. Phytoremediation of petroleum hydrocarbons in tropical coastal soils. II. Microbial response to plant roots and contaminant. Environ Science Pollution Research Vol. 11 (2004) : 340-346.

Jordahl, J.L., Foster, L., Schnoor, J.L., and Alvarez, P.J.J. Effect of hybrid poplar trees on microbial populations important to hazardous waste bioremediation. Environmental Toxicology and Chemistry Vol. 16, No. 6 (1997) : 1318-1321.

Kanaly, R., Bartha, R., Fogel, S., and Findlay, M. Biodegradation of [^{14}C]benzo[*a*]pyrene added in crude oil to uncontaminated soil. Applied and Environmental Microbiology Vol. 63, No. 11 (1997) : 4511-4515.

Karthikeyan, R., Davis, L.C., Mankin, K.R., Erickson, L.E., and Kulakow, P.A. Biodegradation of Jet Fuel (JP-8) in the presence of vegetation. Proceedings of the 1999 conference on hazardous waste research, 243-256. St. Louis, Missouri, 1999.

Keck, J., Sims, R.C., Coover, M., Park, K., and Symons, B. Evidence for cooxidation of polynuclear aromatic hydrocarbons in soil. Water Research Vol. 23, No. 12 (1989) : 1467-1476.

Khan, A. G. Mycorrhizoremediation-an enhanced form of phytoremediation. Journal of Zhejiang University Science B Vol. 7, No.7 (2006) : 503-514.

Komisar, S.J., and Park, J. Phytoremediation of diesel-contaminated soil using alfalfa. Fourth International In Situ and On-Site Bioremediation Symposium, 31-336. New Orleans, LA, 1997.

Koske, R.G., Gemma, J.N., and Englander. Vesicular arbuscular mycorrhizae in *Hawaiian ericales*. Amer. J. Bot., Vol. 77, No.1 (2000) : 64-68

Kuiper, I., Lagendijk, E.L., Bloemberg, G.V., and Lugtenberg, B.J.J. Rhizoremediation: a benefiacial plant-microbe interaction. Molecular Plant-Microbe Interactions Vol. 17 (2004) : 6-15.

Lasat, M.M., Baker, A.J.M., and Kochian, L.V. Physiological characterization of root absorption and translocation to shoots in hyperaccumulator and nonaccumulator species of Thlaspi. Plant physiology Vol. 112, (2000) : 1715 – 1722.

Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Prome', J.C. and De'narie', J. Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. Nature, Vol. 344, (1990) : 781–784.

Liste, H.H., and Alexander, M. Accumulation of phenanthrene and pyrene in rhizosphere soil. Chemosphere Vol. 40 (2000) : 11-14.

Long, S.R. Rhizobium symbiosis: Nod factors in perspective. Plant Cell Vol. 8 (1996) : 1885–1898.

Lugtenberg, B.J.J., and Dekkers, L.C. What makes Pseudomonas bacteria rhizosphere competent. Environmental Microbiology Vol. 1, No. 1 (1999) : 9-13.

Marschner, P., Baumann, K. Changes in bacterial community structure induced by mycorrhizal colonization in split-root maize. Plant and Soil Vol. 251, No. 2 (2003) : 279-289.

Mayo, K., Davis, R., and Motta, J. Stimulation of germination of spores of *Glomus versiforme* by spore-associated bacteria. Mycologia Vol. 78, No.3 (1986) : 426-431.

Merryweather, J.W., and Fitter, A.H. A modified procedure for elucidating the structure of the fungal partner in a vesicular-arbuscular mycorrhiza. Mycological Research Vol. 95 (1991) : 1435-1437.

Morton, J.B., and Benny, G.L. Revised classification of arbuscular mycorrhizal fungi (*Zygomycetes*): A new order, *Glomales*, two new suborders, *Aculosporaceae* and *Gigasporaceae* and *Gigasporaceae*, with an emendation of Glomaceae. Mycotaxon Vol. 37 (1990) : 471-491.

Morton, J.B., Franke, M., and Bentivenga, S.P. Developmental foundations for morphological diversity among endomycorrhizal fungi in Glomales (Zygomycetes). Mycorrhiza: Structure, Function, Molecular Biology, and Biotechnology, 669-683. Springer-Verlag, Berlin : Varma, A. and B. Hock, eds., 1994.

Mosse, B., Powell, C.L. and Hayman, D.S., Plant growth responses to vesicular–arbuscular mycorrhiza. Interactions between VA mycorrhiza, rock phosphate and symbiotic nitrogen fixation. New Phytologist Vol. 76 (1976) : 331–342.

Nagahashi, G., and Douds, D.D. Appressorium formation by AM fungi on isolated cell walls of carrot roots. New Phytologist Vol. 136 (1997) : 299–304.

Niebel, A., Bono, J.J., Ranjeva, R., and Cullimore, J.V. Identification of a high affinity binding site for lipooligosaccharidic NodRm factors in microsomal fraction of Medicago cell suspension cultures. Molecular Plant-Microbe Interactions Vol. 10 (1997) : 132–134.

Paul, E.A., and Clark, F.E. Occurrences and distribution of soil organics. Soil Microbiology and Biochemistry, 81-84. San Diego : E. A. Paul and F. E. Clark. Academic Press, 1989.

Paterson, S., Mackay, D., and McFarlane, C. A model of organic chemical uptake by plants from soil and the atmosphere. Environmental Science and Technology Vol. 28, No. 13 (1994) : 2259-2266.

Phillips, D.A., and Streit, W.R. Legume signals to rhizobial symbionts: a new approach for defining rhizosphere colonization. Plant–Microbe Interactions, 236-271. New York : Chapman & Hall, 1996.

Phillip, J.M., and Hayman, D.S. Improved procedure for clearing root and staining parasite and vesicular arbuscular mycorrhizal fungi for rapid assessment of infections. Transactions of the British Mycological Society Vol. 55 (1970) : 158–160.

Pierson, L.S., Pierson, E.A., and Morello, J.E.. Positive and Negative Cross-communication among Rhizobacteria. In: Leong, S.A., Allen, C., Triplett, E.W. (Eds.), Biology of Plant-microbe Interactions, 256-262. St Paul, MN, USA : International Society for Molecular Plant-microbe Interactions, 2002.

Pothuluri, J.V., and Cerniglia, C.E. Microbial metabolism of polycyclic aromatic hydrocarbons. Biological Degradation and Bioremediation of Toxic Chemicals, 92-124. Portland, Oregon : G. R. Chaudry. Dioscorides Press, 1994.

Pradhan, S.P., Conrad, J.R., Paterek, J.R., and Srivastava, V.J. Potential of phytoremediation for treatment of PAHs in soil at MGP sites. Journal of Soil Contamination Vol. 7, No. 4 (1998) : 467-480.

Price, N.P.J. Broad-host-range Rhizobium species strain NGR234 secretes a family of carbamoylated, and fucosylated, nodulation signals that are O-acetylated or sulfated. Molecular Microbiology Vol. 6 (1992) : 3575–3584.

Qiu, X., Leland, T.W., Shah, S.I., Sorensen, D.L. and Kendall, E.W. Chapter 14 Field study: grass remediation for clay soil contaminated with polycyclic aromatic hydrocarbons. Phytoremediation of soil and water contaminants, 107-112. Washington, D.C. : E.L. Kruger, T.A. Anderson and J.R. Coats. ACS Symposium Series 664, 1997.

Radwan, S.S., Al-Awadhi, H., and El-Nemr, I.M. Cropping as a phytoremediation practice for oily desert soil with reference to crop safety as food. International Journal of Phytoremediation Vol. 2, No.4 (2000) : 383–396.

Radwan, S.S., Al-Awadhi, H., Sorkhoh, N.A., and El-Nemr, I.M. Rhizospheric hydrocarbonutilizing microorganisms as potential contributors to phytoremediation for the oily Kuwaiti desert. Microbiological Research Vol. 153, No. 3 (1998) : 247–251.

Radwan, S.S., Sorkhoh, N. and El-Nemr, I.M. Oil biodegradation around roots. Nature Vol. 376 (1995) : 302.

Redente, E.F., and Reeves, F.B., Interactions between vesicular–arbuscular mycorrhiza and rhizobium and their effect on sweetvetch growth. Soil Science Society of America Vol. 132, No.6 (1981) : 410–415.

Reilly, K.A., Banks, M.K., and Schwab, A.P. Dissipation of polycyclic aromatic hydrocarbons in the rhizosphere. Journal of Environmental Quality Vol. 25 (1996) : 212–219.

Requena, N., Jimenez, I., Toro, M., and Barea, J.M. Interactions between plant-growth-promoting rhizobacteria (PGPR), arbuscular mycorrhizal fungi and *Rhizobium* sp. in the rhizosphere of *Anthllis cytisoides*, a model legume for revegetation in mediterranean semi-arid ecosystems. New Phytologist Vol. 136 (1997) : 667-677.

Reynolds, C.M., and Wolf, D.C. Microbial based strategies for assessing rhizosphereenhanced phytoremediation. Proceedings of the Phytoremediation Technical Seminar, May 31-June 1, 1999, 125-135. Ottawa, Canada : Calgary, AB. Environment, 1999.

Roche, P., Debelle, F., Maillet, F., Lerouge, P., Faucher, C., Truchet, G., Denarie, J., and Prome, J.C. Molecular basis of symbiotic host specificity in Rhizobium meliloti: nodH and nodPQ genes encode the sulfatation of lipo-oligosaccharides signals. Cell Vol. 67 (1991) : 1131–1143.

Rodriguez, H., and Fraga, R. Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnology Advances Vol. 17 No. 4-5 (1999) : 319-339.

Rousseau, J.V., Sylvia, D.M., and Fox A.J. Contribution of mycorrhiza to the potential nutrient-absorbing surface of pine. New Phytologist Vol. 128 (1994) : 639-644.

Salt, D.E., Smith, R.D., and Raskin, I. Phytoremediation. Annual Review of Plant Physiology and Plant Molecular Biology No. 49 (1998) : 643-668.

Schnoor, J.L., Licht, L.A., McCutcheon, S.C., Wolfe, N.L., and Carreira, L.H. Phytoremediation of organic and nutrient contaminants. Environmental Science and Technology Vol. 29, No. 7 (1995) : 318-323.

Schwab, A.P., Banks, M.K., and Arunachalam, M. Biodegradation of polycyclic aromatic hydrocarbons in rhizosphere soil. Bioremediation of Recalcitrant Organics, 23-29. Columbus : Battelle Press, 1995.

Schwab, A.P., Su, J., Wetzel, S., Pekarek, S., and Banks, M.K.,. Extraction of petroleum hydrocarbons from soil by mechanical shaking. Environmental Science and Technology Vol. 33 (1999) : 1940–1945.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Schwarzott, D., and Arthur, S. A simple and reliable method for SSU rRNA gene DNA extraction, amplification, and cloning from single AMF spores. Mycorrhiza Vol. 10 (2001) : 203-207.

Schwendinger, R.B. Reclamation of soil contaminated with oil. Journal of the Institute of Petroleum Vol. 54 (1968) : 182-197.

Shinkarev, V.P., Brunner, R., White, J.O., and Wraight, C.A. The structure of chromatophores from purple photosynthetic bacteria fused with lipid-impregnated collodion films determined by near-field scanning optical microscopy. FEBS Letters Vol. 452 (1999) : 223–227.

Siciliano, S.D., and Germida, J.J. Degradation of chlorinated benzoic acid mixtures by plantbacteria associations. Environmental Toxicology and Chemistry Vol. 17, No. 4 (1998) : 728-733.

Siciliano, S.D., Germida, J.J., and Siciliano, S.D. Biology analysis and fatty acid methyl ester profiles indicate that pseudomonad inoculants that promote phytoremediation alter the root-associated microbial community of *Bromus biebersteinii*. Soil biology and biochemistry Vol. 30 (1998) : 1717–1723.

Sims, R.C., and Overcash, M.R. Fate of polynuclear aromatic compounds (PNAs) in soilplant systems. Residue Reviews Vol. 88 (1983) : 1-68.

Siqueira, J.O., Carneiro, M.A.C., Curi, N., Rosado, S.C.S., and Davide, A.C. Mycorrhizal colonization and mycotrophic growth of native woody species as related to successional groups in southeastern Brazil. Forest Ecology and Management Vol.107 (1998) : 241–252.

Smith, F.A. and Smith, S.E. Structural diversity in (vesicular)–arbuscular mycorrhizal symbioses. New Phytologist Vol.137 (1997) : 373–388.

Smith, S. E. Discoveries, discussions and directions in research on mycorrhizae. Mycorrhiza: Function, Molecular Biology and Biotechnology, 3-24. Springer Verlag, 1995.

Siu, T.T., Anthony, D.W., and Kjell, U. Detailed hydrocarbon analysis of gasoline by GC-MS (SI-PIONA). Journal of High Resolution Chromatography Vol. 17, No. 6 (2005) : 469-475.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Sutton, J.M., Lea, E.J.A., and Downie, J.A. The nodulation-signaling protein nodO from Rhizobium leguminosarum biovar viciae forms ion channels in membranes. Proceedings of the National Academy of Sciences Vol. 91 (1994) : 9990–9994.

Sutherland, J.B. Detoxification of polycyclic aromatic hydrocarbons by fungi. Journal of Industrial Microbiology Vol. 9 (1992) : 53-62.

Tollsten, L., and Muller, P. Volatile organic compounds emitted from beech leaves. Phytochemistry Vol. 43 (1996) : 759-762.

Trapp, S., Matthies, M., Scheunert, I., and Topp, E. M. Modeling the bioconcentration of organic chemicals in plants. Environmental Science and Technology. Vol. 24, No. 8 (1990) : 1246-1252.

Turgeon, B.G., and Bauer, W.D. Ultrastructure of infection-thread development during infection of soybean by Rhizobium japonicum. Planta Vol. 163 (1985) : 328–349.

Van Brussel, A.A.N., Bakhuizen, R., Van Spronsen, P.C., Spaink, H.P., Tak, T., Lugtenberg, B.J.J., and Kijne, J.W. Induction of preinfection thread structures in the leguminous host plant by mitogenic lipo-oligosaccharides of Rhizobium. Science Vol. 257 (1992) : 70–71.

Van Rhijn, P., Goldberg, R.B., and Hirsch, A.M. Lotus corniculatus nodulation specificity is changed by the presence of a soybean lectin gene. Plant Cell Vol. 10 (1998) : 1233–1249.

Vance, D.B. Phytoremediation: enhancing natural attunation processes. National Environmental Journal Vol. 6 (1996) : 30-31.

Venosa, A., Suidan, M., Wrenn, B., Strohmeier, K.L., Haines, J.R., Eberhart, B.L., King, D., and Holder, E. Bioremediation of an experimental oil spill on the shoreline of Delaware Bay. Environmental Science and Technology Vol. 30 (1996) : 1764–1775.

Vivas, A., Marulanda, A., Ruiz-Lozana, J.M., Barea, J.M., and Azcon, R. Influence of a Bacillus sp. on physiological activities of two arbuscular mycorrhizal fungi and on plant responses to PEG-induced drought stress. Mycorrhiza Vol. 13, No. 5 (2003) : 249-256.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Vijn, I., Martinez-Abarca, F., Yang, W.C., Das Neves, L., van Brussel, A., Van Kammen, A., and Bisseling,T. Early nodulin gene expression during Nod factor-induced processes in Vicia sativa. The Plant Journal Vol. 8 (1995) : 111–119.

Vlassak, K.M., Luyten, E., Verreth, C., Van Rhijn, P., Bisseling, T., and Vanderleyden, J. The Rhizobium sp. BR816 nodO gene can function as a determinant for nodulation of Leucaena leucocephala, Phaseolus vulgaris, and Trifolium repens by a diversity of Rhizobium spp. Molecular Plant-Microbe Interactions Vol. 11 (1998) : 383–392.

Vouillamoz, J., and Milke, M.W. Effect of compost in phytoremediation of diesel-contaminated soils. Water Science and Technology Vol. 43, No.2 (2001) : 291–295.

Wang, X., and Bartha, R. Remediation of Hazardous Waste Contaminated Soils. New York : Wise, 1994.

Walker, C., and Trappe, J. M. Names and epithets in the Glomales and Endogonales. Mycological Research Vol. 97 (1993) : 339-344.

Walton, B.T., Guthrie, W.A., and Hoylman, A.M. Toxicant degradation in the rhizosphere. T.A. Anderson and J.R. Coats. Bioremediation Through Rhizosphere Technology, 11-26. Washington D.C. : American Chemical Society, 1994.

Watkins, J.W., Sorensen, D.L., and Sims, R.C. Chapter 11 Volatilization and mineralization of naphthalene in soil-grass microcosms. Bioremediation Through Rhizosphere Technology, 123-131. Washinton, D.C. : T.A. Anderson and J.R. Coats. ACS Symposium Series 563, 1994.

Wetzel, S.C., Banks, M.K., and Schwab, A.P. Chapter 18 Rhizosphere effects on the degradation of pyrene and anthracene in soil. Phytoremediation of soil and water contaminants, 254-262. Washington, D.C. : E.L. Krúger, T.A. Anderson and J.R. Coats. ACS Symposium Series 664, 1997.

Wiltse, C.C., Rooney, W.L., Chen, Z., Schwab, A.P., and Banks, M.K. Greenhouse evaluation of agronomic and crude oil-phytoremediation potential among alfalfa genotypes. Journal of Environmental Quality Vol. 27, No. 1 (1998) : 169–173.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

Xu, J.G., and Johnson, R.L. Nitrogen dynamics in soils with different hydrocarbon contents planted to barley and field pea. Canadian Journal of Soil Science Vol. 77 (1997) : 453-458.

Yang, W.C., De Blank, C., Meskiene, I., Hirt, H., Bakker, J., Van Kammen, A., Franssen, H., and Bisseling, T. Rhizobium nod factors reactivate the cell cycle during infection and nodule primordium formation, but the cycle is only completed in primordium formation. Plant Cell Vol. 6 (1994) : 1415–1426.

Zhang, S., Reddy, M.S., and Kloepper, J.W. Development of assay for assessing induced resistance by plant-growth-promoting-rhizobacteria against blue mold of tobacco. Biological Control Vol. 23, No. 1 (2002) : 79-86.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

APPENDICES

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX A

Taxonomy and identification of arbuscular mycorrhizal fungi in genera level (Bernhard, 2002)

Taxonomy	Identification
Suborder Glomineae	forms intraradicle vesicles
Family Glomaceae	Generally has simple wall structure, inner walls do not react in Melzer's reagent (iodine)
Genus *Glomus*	spores borne singly or in sporocarps; subtending hypha present, straight, flared or recurved
Genus *Scelerocystis*	obligately sporocarpic, spores organized radially around a central plexus of hyphae
Family Acaulosporaceae	More complex wall structure (4-6 walls), inner walls may react with Melzer's; outer wall may be ornamented; Spore borne in or on neck of saccule
Genus *Acaulospora*	Spore borne on saccule neck; one scar in mature spores
Genus *Entrophospora*	Spore borne in saccule neck; two scares in mature spores
Suborder Gigasporineae	No intraradicle vesicles; huge spores (250 um); bulbous suspensor cell
Family Gigasporaceae	The spore contents are partitioned from that of the bulbous sporogenous cell by a plug or, more rarely, by a septum
Genus *Gigaspora*	Inner walls present, may react with Melzer's; germination shield absence
Genus *Scutellospora*	Inner walls present, may react with Melzer's; germination shield present

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX B

Modified Bell's nutrient solution (Gazey et al. 1992)

Compound	Amount (g/l)	Solution
KH_2PO_4	10.8	A
K_2SO_4	45	
NH_4NO_3	15	
$CaCl_2.2H_2O$	45	B
$MgSO_4.7H_2O$	6	C
$MnSO_4.H_2O$	3	
$ZnSO_4.7H_2O$	3	
$CuSO_4.5H_2O$	1.5	
H_3BO_3	0.24	
$CoSO_4.7H_2O$	0.12	
$Na_2MoO_4.2H_2O$	0.09	

These nutrients are dissolved in water to form three stock solutions (A, B, and C) which are further diluted into a nutrient solution by adding 33 ml of each stock/l of final volume. This solution is applied to soil by watering

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX C

Wet sieving and decanting technique (Gerdemann and Nicolson, 1963)

1. Approximately 200g of soil were suspended in one liter of water. Heavier particles were allowed to settle for a few seconds and the liquid was decanted through a sieve fine enough to remove the larger particles of organic matter, but coarse enough to allow the desired spores to pass through such as two mm sieve.
2. The suspension that passed through this sieve was saved and stirred to resuspend all particles. The heavier particles were allowed to settle for a few seconds and the liquid decanted again through the sieve, ensure that soil aggregates have broken apart.
3. This washing and decanting process was repeated until the water was clear.
4. Vigorous washing with water and hand mixing were necessary to free spores from aggregates of clay or organic materials.
5. The sievings retained on different sieves were washed into separate petri dishes for further observations or purification by sucrose centrifugation.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX D

Sucrose centrifugation (Daniel and Skipper, 1979)

1. Spores and minimal amount of organic particles could be purified by suspending sievings in the 40% sucrose solution and centrifugation at 2000 rpm (approximate 370 x g) for 1 minute to separate spores from denser soil components.
2. The supernatant (with spores) was poured through the sieve of 400 mesh and rinsed with distilled water to remove sucrose.
3. The isolated AM spores were soaked in 2 % chloramin T mixing with 0.05 % Tween 20 for 20 min.
4. Spores were washed 3 times in sterile distilled water then soaked in 200 ug/ml streptomycin and 100 ug/ml gentamycin for 20 min.

APPENDIX E

Roots clearing and staining method (Philips and Hayman, 1970)

1. Cut roots into 2 – 4cm long segments before staining for uniform contact with solution.
2. 10% w/v KOH was used to clear roots by autoclaving at 121°C for 20 minutes.
3. Cleared roots were captured on a fine sieve and rinsed with water before transferring them into the staining solution.
4. Stained with 0.05% w/v of trypan blue in lactoglycerol at 121°C for 15 minutes in autoclave.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX F

Gridline intersection method (Giovannetti et.al. 1994)

1. Randomly disperse cleared and stained roots in a dish with grid lines.
2. Assess mycorrhizal colonisation under a dissecting microscope.
3. Follow all horizontal and vertical lines, count intersects with roots and mycorrhizas separately.
4. The ration of mycorrhiza intersects over root intersects is the %AM infection.
5. The subsamples of roots were randomly selected to determine under compound microscope to recognize the fungi morphology.

APPENDIX G

Spore staining and permanent slide mountant (Koske and Gemma, 2000)

1. After isolate the spores from fresh pot culture, a minimum of 20 spores per each host plant were collected in glass watch.
2. These spores were analyzed in both forms of intact and crushed after mounted with PVLG (polyvinyl lactoglycerol) and Melzer's PVLG (INVAM, 1997).

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX H

Spore mounting media

1. Polyvinyl alcohol-lacto-glycerol (PVLG) mountant (Koske and Gemma, 2000)

Polyvinyl alcohol 8.33 g.

Distilled water 50 ml.

Lactic acid 50 ml.

Glycerine 5 ml.

2. Melzer's reagent (Morton, 1990)

Iodine 1.5 g.

Potassium iodide 5 g.

Distilled water 100 ml.

Mixed 1:1 (v/v) of PVLG:Melzer's reagent

APPENDIX I

Taxonomy of arbuscular mycorrhizal fungi in genera level (Bernhard, 2002)

Taxonomy	Identification
Suborder Glomineae	forms intraradicle vesicles
Family Glomaceae	Generally has simple wall structure, inner walls do not react in Melzer's reagent (iodine)
Genus *Glomus*	spores borne singly or in sporocarps; subtending hypha present, straight, flared or recurved
Genus *Scelerocystis*	obligately sporocarpic, spores organized radially around a central plexus of hyphae
Family Acaulosporaceae	More complex wall structure (4-6 walls), inner walls may react with Melzer's; outer wall may be ornamented; Spore borne in or on neck of saccule
Genus *Acaulospora*	Spore borne on saccule neck; one scar in mature spores
Genus *Entrophospora*	Spore borne in saccule neck; two scares in mature spores
Suborder Gigasporineae	No intraradicle vesicles; huge spores (250 um); bulbous suspensor cell
Family Gigasporaceae	The spore contents are partitioned from that of the bulbous sporogenous cell by a plug or, more rarely, by a septum
Genus *Gigaspora*	Inner walls present, may react with Melzer's; germination shield absence
Genus *Scutellospora*	Inner walls present, may react with Melzer's; germination shield present

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

APPENDIX J

Randomized complete block design in biostimulation experiment

Experiment	Treatment*	Jack bean	Rhizobium fertilizer (g)	AM (spores)
1	P	✓	-	-
2	R+P	✓	10	-
3	A10+P	✓	-	10
4	A20+P	✓	-	20
5	A50+P	✓	-	50
6	A100+P	✓	-	100
7	R+A10+P	✓	10	10
8	R+A20+P	✓	10	20
9	R+A50+P	✓	10	50
10	R+A100+P	✓	10	100

* P: plant without inoculation; R+P: 10g rhizobium with plant; A10+P: 10 spores AM with plant; A20+P: 20 spores AM with plant; A50+P: 50 spores AM with plant; A100+P: 100 spores AM with plant; R+A10+P: 10g rhizobium and 10 spores AM with plant; R+A20+P: 10g rhizobium and 20 spores AM with plant; R+A50+P: 10g rhizobium and 50 spores AM with plant; R+A100+P: 10g rhizobium and 100 spores AM with plant

10 treatments with 3 replicates is randomized as followed,

Block 1	10	3	7	1	8	2	4	6	9	5
Block 2	7	1	8	10	6	9	4	2	5	3
Block 3	8	3	4	5	9	7	2	1	10	6

APPENDIX K

Randomized complete block design in phytoremediation experiment

Experiment	Treatment*	Rhizobium fertilizer (g)	AM (spores)	Jack bean	Diesel oil
11	P'	-	-	✓	-
12	R+P'	10	-	✓	-
13	A+P'	-	100	✓	-
14	R+A+P'	10	100	✓	-
15	NP	-	-	-	✓
16	P	-	-	✓	✓
17	R+P	10	-	✓	✓
18	A+P	-	100	✓	✓
19	R+A+P	10	100	✓	✓

* P': plant without inoculation; P: plant without inoculation with oil; R+P: 10g rhizobium with plant; A+P: 100 spores AM with plant; R+A+P: 10g rhizobium and 100 spores AM with plant

9 treatments with 3 replicates is randomized as followed,

Block 1	1	8	3	6	5	9	2	4	7
Block 2	9	2	1	4	3	7	5	8	6
Block 3	3	6	9	2	5	8	1	4	7

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย

CURRICULUM VITAE

Ms.Siriyaporn Juthapruth was born in Bangkok, Thailand, on April 29, 1982. She received a Bachelor Degree of Science in Botany, Mahidol University in 2004. Then, she continued her post-graduate study in Botany Major at the Department of Botany, Faculty of Science, Chulalongkorn University. During her Master degree enrollment, she presented research work in the 20th Annual Meeting and International Conference of the Thai Society for Biotechnology in 2008 at Taksila Hotel, Maha Sarakham, Thailand. She ultimately completed the degree of Master of Science in Botany in May 2009.

ศูนย์วิทยทรัพยากร
จุฬาลงกรณ์มหาวิทยาลัย