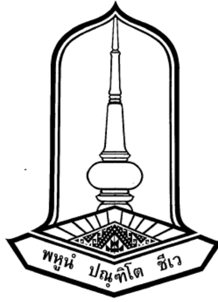

IMPACT OF HEAVY METAL CONTAMINATIONS IN AGRICULTURAL SOIL ON ZEA MAYS L. RHIZOBACTERIAL COMMUNITY AND ROOT EXTRACTS

NATTHAWOOT PANITLERTUMPAI

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor Philosophy in Biology at Mahasarakham University

June 2017

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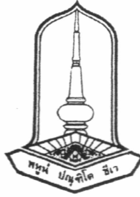

The examining committee has unanimously approved this dissertation, submitted by Mr. Natthawoot Panitlertumpai, as a partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biology at Mahasarakham University.

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ACKNOWLEDGEMENTS

This thesis was funded by the Junior Science Talent Project and the National Science and Technology Development Agency (NSTDA) with the grant number of JSTP-06-54-02E.

I appreciate Assoc. Prof. Dr. Paitip Thiravetyan, School of Bioresources and Technology, King Mongkut's University of Technology Thonburi (KMUTT) for valuable suggestions and comments. I am particularly grateful to Assist. Prof. Dr. Khanitta Somtrakoon, Dr. Akeapot Srifa, and Assoc. Prof. Dr. Aphidech Sangdee, Department of Science, Faculty of Biology, Mahasarakham University (MSU), for valuable suggestions. I would like to express my sincere and deep gratitude to Assist. Prof. Dr. Woranan Nakbanpote, my thesis advisor, for her enlightening suggestions, untiring support and affectionate encouragement through this study.

I would also like to thank the Faculty of Science and Technology, Rajabhat Mahasarakham University and Land Development Office of Region 5, Khon Kaen, Thailand for research facilities of inductively coupled plasma optical emission spectrometry (ICP-OES) and soil analysis, respectively.

I am deeply indebted to Prof. Dr. Neung Teaumroong, School of Biotechnology, Institute of Agricultural Technology, Suranaree University of Technology for valuable suggestions and supporting in PCR-DGGE (Polymerase Chain Reaction Denaturing Gradient Gel Electrophoresis), and sincerely thank Nitra Nuengchamnong, Science Laboratory Centre, Faculty of Science, Naresuan University for her suggestion in LC-ESIQTOF-MS/Ms analysis. I am grateful to Dr. Jolyon Dodgson for English proofreading of both this thesis and manuscripts for publication.

This thesis would not have been possible without assistance and willpower from my parents. I would like to thank all my friends for their friendship and help during y study, especially the members of the Sustainable Development Laboratory (SDL). Finally, I gratefully thank the officers at the Faculty of Science and Faculty of Graduate Studies, MSU for coordination and good activities.

Mr. Natthawoot Panitlertumpai

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ชื่อเรื่อง ผลกระทบของโลหะหนักที่ปนเปื้อนอยู่ในดินทำการเกษตร ต่อสังคมจุลินทรีย์รอบรากและสารสกัดจากรากของต้นข้าวโพด
ผู้วิจัย นายนัฐวุฒิ พานิชย์เลิศอำไพ
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บทคัดย่อ

พื้นที่ปลูกข้าวโพดติดลำน้ำแม่ตาว หมู่บ้านพะเด๊ะ อำเภอแม่สอด จังหวัดตาก ประสบปัญหาปนเปื้อนโลหะสังกะสี แคดเมียม และตะกั่ว งานวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาความสัมพันธ์ระหว่างระดับการปนเปื้อนของโลหะในดินกับการเติบโตของต้นข้าวโพด การสะสมโลหะหนักในเมล็ด และความหลากหลายของแบคทีเรียในดินรวมและดินรอบรากข้าวโพด โดยเก็บตัวอย่างข้าวโพดพันธุ์ลูกผสมทีเอฟ 222 ซึ่งปลูกตามวิธีของชาวบ้านเป็นเวลา 4 เดือน (7 มิถุนายน-7 ตุลาคม 2556) ในแปลงขนาด (1.5x10 ตารางเมตร) จำนวน 5 แปลงติดต่อกัน มีปริมาณโลหะหนักไล่ระดับจากสูงบริเวณใกล้ลำน้ำและลดลงเมื่อลึกเข้าไปในที่ดิน คือ สังกะสี 379-4,883 กรัมต่อกิโลกรัม แคดเมียม 6-85 กรัมต่อกิโลกรัม และตะกั่ว 34-154 กรัมต่อกิโลกรัม ใช้วิธีมาตรฐานในการวิเคราะห์ดินและพืช ศึกษาความหลากหลายของแบคทีเรียที่เลี้ยงได้โดยใช้อาหาร Nutrient agar (NA) และศึกษาความหลากหลายของแบคทีเรียโดยรวมด้วยวิธี PCR-DGGE ผลการศึกษาแสดงว่า ปริมาณน้ำฝนและการไหลบ่าของน้ำในพื้นที่เพาะปลูกเป็นตัวแปรที่ส่งผลต่อปริมาณโลหะหนักในรูปที่พืชนำไปใช้ได้ ความหลากหลายของแบคทีเรียในดินรอบรากข้าวโพดเป็นตัวแปรที่ส่งผลต่อปริมาณการสะสมของโลหะสังกะสี และแคดเมียมของข้าวโพด ระดับการปนเปื้อนของโลหะหนักในดินที่แตกต่างกันไม่ส่งผลต่อความหลากหลายของแบคทีเรียในดินรอบรากข้าวโพด แต่ส่งผลต่อความหลากหลายของแบคทีเรียโดยรวม โดย *Brevibacillus agri* (KY618802) *Bacillus* sp. (KY629623 และ KY629626) *Cellulosimicrobium funkei* (KY629624) และ *Pseudomonas chlororaphis* (KY629627) เป็นเชื้อที่พบในทุกระดับการปนเปื้อนของโลหะในดินรอบรากข้าวโพดและทุกการเติบโตของต้นข้าวโพด นอกจากนั้นข้าวโพดพันธุ์ที่ปลูกในพื้นที่สามารถทนต่อโลหะหนักและสะสมสังกะสี โดยพบสังกะสีในเมล็ดข้าวโพด 11-30 มิลลิกรัมต่อกิโลกรัม ตามคุณค่าทางโภชนาการ (เกณฑ์มาตรฐาน 16.5-24.6 มิลลิกรัมต่อกิโลกรัม) ส่วนแคดเมียมและตะกั่วในเมล็ดอยู่ในระดับต่ำกว่าขีดจำกัดการวัดเชิงปริมาณ ภายหลังจากเก็บเกี่ยวดัชนีมลพิษและดัชนีความเสี่ยงทางนิเวศมีค่าลดลง ผลการศึกษาในภาคสนามภายใต้สภาพแวดล้อมของพื้นที่จริงสามารถเป็นข้อมูลเพื่อใช้บริหารจัดการการเพาะปลูกพืชในพื้นที่โดยเฉพาะข้าวโพด

สารสกัดจากรากข้าวโพดอายุ 1 ถึง 4 สัปดาห์ ที่ปลูกในกระถางทดลองบรรจุดินตัวอย่างจากพื้นที่ปลูกข้าวโพดแปลงที่ 1, 3 และ 5 ซึ่งปนเปื้อนโลหะ สังกะสี แคดเมียม และตะกั่ว เรียง 3 ระดับ คือ สูง กลาง และต่ำ ผลการศึกษาเทียบกับสารสกัดจากรากข้าวโพดที่ปลูกในกระถางบรรจุดินไม่ปนเปื้อนโลหะหนักพบว่า สารสกัดจากรากข้าวโพดประกอบด้วยสารประกอบในกลุ่มฟีนอลลิก ฟลาโวนอยด์ กรดอินทรีย์ และอนุพันธ์ที่มีน้ำตาลเป็นองค์ประกอบ และการวิเคราะห์ทางสถิติพบว่าสารสกัดจากราก

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ข้าวโพดเป็นตัวแปรที่เกี่ยวข้องกับกลไกการทนโลหะหนักและการสะสมโลหะหนักในต้นข้าวโพด และการละลายของโลหะหนักในดิน โดยสารประกอบฟีนอลิก และสารประกอบฟลาโวนอยด์ เช่น คาเทชิน กรดแกลลิค วานิลลิน และกรดพีคูมาริก เป็นตัวแปรที่ส่งผลต่อการเติบโตและการสะสมโลหะสังกะสีและแคดเมียม สารประกอบกรดอินทรีย์ เช่น กรดออกซาลิก กรดมาลิก กรดมาเลอิก กรดซิตริก และกรดซักซินิก เป็นตัวแปรที่ส่งผลต่อการเติบโต นอกจากนี้ ออกซาลิกยังส่งผลต่อปริมาณการสะสมของโลหะสังกะสีและแคดเมียม ส่วนกรดมาเลอิกและกรดซิตริกส่งผลต่อการละลายของโลหะสังกะสีในดิน ทั้งนี้ กลไกของสารประกอบฟีนอลิก สารประกอบฟลาโวนอยด์ และกรดอินทรีย์ในรากต้นข้าวโพดที่สัมพันธ์กับชนิดและปริมาณของโลหะหนักควรมีการศึกษาเพิ่มเติมต่อไป

คำสำคัญ: การทดลองภาคสนาม ดินปนเปื้อนโลหะหนัก สังคมจุลินทรีย์ สารสกัดจากราก ข้าวโพด

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TITLE Impact of heavy metal contaminations in agricultural soil on *Zea mays* L. rhizobacterial community and root extracts

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DEGREE Doctor Philosophy **MAJOR** Biology

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UNIVERSITY Mahasarakham University **YEAR** 2017

ABSTRACT

A maize field near Mae Tao Creek in Ban Pha Te, Mae Sot District, Tak Province has been reported as a Zn, Cd, and Pb polluted area. This research aims to study the interaction between the levels of the metals contaminated soil and maize development, heavy metals accumulated in seed, and bacterial community. The field experiment of hybrid maize seeds TF 222 was carried out for four months (June 7 to October 7, 2013) by following conservation planting practices. A plot experiment was divided into five consecutive portions of 1.5 m x 10 m, in which the metals contents were gradual from high near the canal and reduced when deeper into the land, as Zn 380-4,883 mg kg^{-1}, Cd 6-85 mg kg^{-1}, and Pb 34-154 mg kg^{-1}. Standard methods were performed to study soil properties and plant analysis. The community of culturable bacteria was studied on nutrient agar (NA). The total bacterial community were studied by PCR-DGGE. Our results showed that rainfall and irrigation were the main factors for the bioavailable of Zn, Cd, and Pb in the field. The changing contents of the heavy metals did not affect the culturable rhizobacterial community, but the contents affected the overall diversity of bacteria in the rhizosphere. *Brevibacillus agri* (KY618802), *Bacillus* sp. (KY629623 and KY629626), *Cellulosimicrobium funkei* (KY629624), and *Pseudomonas chlororaphis* (KY629627) were found in all the levels of the metals contaminated soil and maize developments. In addition, the maize tolerated the heavy metals and accumulated a high Zn in its biomass. The Zn content in their maize seeds was 11-30 mg kg^{-1}, which was within the nutritional standard of 16.5-24.6 mg Zn kg^{-1}. While the contents of Cd and Pb in the maize seed were under our limit of detection. After harvesting, the *pollution index*

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and *potential ecological risk index* of the soils were decreased. The results obtained could be information for the management of crop cultivation in the area, especially the maize.

The root extracts from 1 to 4 week-old maize growing in pots filled with three soil samples from plots 1, 3, and 5, which were contaminated with Zn, Cd, and Pb in the three levels of high, middle, and low, respectively. In comparison with the root extracts from maize growing in the non-contaminated soil, the results showed that the root extracts contained phenolic compounds, flavonoids, organic acids, and their derivative of the sugar side chain. Statistical analysis of the pot data indicated that phenolic compounds and flavonoids, such as catechin, gallic acid, vanillin, and *p-coumaric acid,* were the main factors for maize growth and metals (Zn and Cd) accumulation. Organic acids, such as oxalic acid, malic acid, maleic acid, citric acid, and succinic acid, were the main factors for the maize growth. In addition, oxalic acid was involved with Zn and Cd accumulation, whereas maleic acid and citric acid also caused an increase in the bioavailable Zn content in the soil. Consequently, the phenolic compounds, flavonoids, and organic acids in the root extracts of maize were related to the metal types and their content, and should be studied further.

Keywords: Rhizosphere, PCR-DGGE, Heavy metals contaminated soil, Root exudates

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CHAPTER 1

INTRODUCTION

1.1 Background

Metal pollution problems occur when human activities either disrupt normal biogeochemical cycles or concentrate metals, such as industrial activities, agricultural activities, mining, and ore refinement (Kabata-Pendias, 2011; Prasad and Nakbanpote, 2015). Mining activities generate a large amount of waste rock and tailings that are deposited at the surface (Wong, 2003). Metal wastes can exist as individual metals or, more often, as metal mixtures. Agricultural soils in the fields of Phatat Phadaeng sub-district, Mae Sot, Tak Province, Thailand, a source of Zn mineralisation, have been demonstrated to be a heavy metals polluted area, especially by Cd (Akkajit, 2015; Khaokaew and Landrot, 2015). The area is rich in zinc silicate (Hemimorphite $Zn_4(Si_2O_7)(OH)_2.2H_2O$) with a marginal zinc carbonate level (Smithsonite $ZnCO_3$) (Pollution Control Department, 2004; Khaokaew *et al*., 2012). The Cd contamination in Phatat Phadaeng came from natural activity, agricultural activity, and careless mining in the past; the contamination was higher along the edge of the Mae Tao Creek than further inland (Prasad *et al*., 2015). The run off and irrigation through drains and flooded rice fields in that area are the reasons for the heavy metal contamination in the rice fields (Simmons *et al*., 2005; Sebastian *et al*., 2016). Soil samples from agricultural areas near Mae Tao Creek in Pha Te Village have total Cd and Zn concentrations ranging from 0.63 to 30.4 mg Cd kg^{-1} and 14.4 to 594 mg Zn kg^{-1}, respectively (Akkajit, 2015). The concentrations were much higher than the Thai investigation level of contaminated agriculture soil with 70 mg Zn kg^{-1}, 0.15 mg Cd kg^{-1}, and 55 mg Pb kg^{-1}, respectively (Zarcinas *et al*., 2004). About 21.42 km^2 near the mines have been declared as unsafe for growing food crops (Prasad *et al*., 2015). Chemical-physical technologies used for the decontamination of contaminated ecosystems are complex and expensive, and it may cause undesirable side effects for the environment that must be turned on to begin the decontamination process. Thus, phytomanagement and related agronomic practices are considered to be cost effective for this problem (Prasad *et al*., 2015).

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Crop cultivation in metal polluted areas should be carefully performed, and edible crops that are able to accumulate those metals in the food chain must be avoided. In 2004-2006, the Thai Government encouraged farmers to stop rice cultivation and supported the production of non-food crops, such as decorative palm, Para rubber (*Hevea brasiliensis* Muell. Arg), and sugarcane (*Saccharum officinarum* (L.)) for ethanol production (Sriprachote *et al*., 2012). However, sugarcane prices were not high enough to stimulate farmers to grow sugarcane. As a result, inhabitants continued to grow crops such as maize (*Zea mays* L.), one of the five major crops in Thailand (Ekasingh *et al*., 2004), and it occupies a major portion of Thai farmland. When considering shoot to grain translocation of heavy metals, maize produces more shoot and root biomass per unit land area. Maize is considerably high biomass, and it can grow in most environments, especially various heavy metals contaminated soils (Thewys *et al*., 2010; Cheng *et al*., 2015; Prasad *et al*., 2015). While mitigating contamination, biomass production can be considered for renewable energy options and edible crops have as use for animal feed (Thewys *et al*., 2010; Moreira *et al*., 2014; Cheng *et al*., 2015). During 2016-2019, about 38.41% of total agricultural area in Mae Sot (approximately 8,072 acres) supported the growth of maize following the Agricultural Development Plan of the National Farmers Council of Tak. Therefore, the pollution in maize fields, which are used to grow maize in the Mae Sot area needs to be investigated for sustainable development by phytomanagement. It may be possible to treat the gain crops with Cd-tolerant plant growth promoting rhizobacteria, which could promote plant growth and decrease the phytoavailability of Cd in the soil (Prasad and Nakbanpote, 2015).

The phytoavailability of metals is influenced by soil associated factors, such as pH, redox potential, cation exchange capacity, soil type, and soil texture, and plant-associated factors, such as root exudates and root rhizosphere processes (Sheoran *et al*., 2016). The rhizosphere may be defined as the portion of the soil that adheres to the plant root where the biology and chemistry of the soil are influenced by the root (Walker *et al*., 2003). This area has a wide range of soil microorganisms. Plant-microbe symbioses are ubiquitous in natural and most anthropogenically influenced soil (Wang *et al*., 2008). Soil microbial activities are the key components for recycling of nutrients, maintenance of soil structure, detoxification of heavy metals, control of plant pests, and promoting plant growth (Hu *et al*., 2007; Wang *et al*., 2008; Singh *et al*., 2011; Berendsen *et al*., 2012).

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The activities are important factors for successful phytoremediation and phytomanagement. However, the presence of high amounts of heavy metals tremendously affects the activities of soil microorganisms and soil microbial community with possible beneficial effects to the plant-soil association (Wang *et al*., 2008; Siripornadulsil and Siripornadulsil, 2013). A shift in microbial community was shown in Cu-, Zn-, and Cd-contaminated soil (Li *et al*., 2006; Wang *et al*., 2007). Hu *et al*. (2007) explained that the bacterial population in soil was affected by Zn and Pb contaminated soil. On the other hand, Cd contaminated soil with approximately 9 mg kg^{-1} affected the bacterial diversity (Unhalekhaka and Kositanont, 2009).

In addition, root exudates can change the chemical and physical properties of the soil surrounding the rhizosphere, regulate the soil microbial community in their immediate vicinity, and have a detrimental effect on the growth of plant species (Walker *et al*., 2003). Metals toxicity could affect the amount and composition of root exudates (Shi, 2009). For example, a concentration of organic acids in the root exudate of *Pinus sylvestris* significantly increased in soil contaminated with Cd (Ahonen-Jonnarth *et al*., 2000). Dong *et al*. (2007) reported that plant roots excreted some organic compounds to the rhizosphere under heavy metal stress to control the entrance of metals, such as Cd, to the plant. Organic acids can bind heavy metals and decease the heavy metal toxicity (Rauser, 1999; Manara, 2012). In addition, phenolic compounds play an important role in the shape of the rhizosphere microbial community, because phenolic compounds are specific substrates or signaling molecules for a large group of soil microbial species (Huang *et al*., 2014). Metal availability and soluble nutrients in the rhizosphere are influenced by root exudates and microorganisms (Wenzel *et al*., 2004; Carvalhais *et al*., 2010). In addition, root exudation patterns usually change quantitatively and qualitatively following plant development and the location along the root system (Ma, 2000). High concentrations of root exudates were found during the initial growth phase due to seed storage compounds (Hamlen *et al*., 1972). Therefore, understanding the interactions between soil microorganisms and root exudates in the rhizosphere of maize could enhance plant growth and manage heavy metal translocation to shoots with multi-contaminated soil.

Many methods can be applied to investigate the microbial community in the rhizosphere**.** Finding a suitable method for this study is a challenging task, because some

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organism numbers may be too large or too small. Therefore, the methods of enumeration in microorganisms have evolved (Hurst *et al*., 1997). Visualization techniques take advantage of the direct observation of microorganisms in the rhizosphere (Campbell and Rovira, 1973), such as determination of metabolically active bacteria and fungi (Bottomley, 1994), and the application in microbial ecology (Bohlool and Schmidt, 1980). Bacterial communities in the soil around Pb and Zn mines were significantly different in colony morphologies, e.g., color, shape, and size, etc. (Hu *et al*., 2007). On the other hand, molecular techniques, such as 16S ribosomal DNA (16S rDNA) and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE), have been well established as standard methods for the identification of bacterial species and bacterial community in soil (Gürtler and Stanisich, 1996; Kozdrój and Elsas, 2000; Li *et al*., 2006; Ahn *et al.*, 2009). These methods were suitable to study the microbial community in heavy metal contaminated soil, such as with Zn, Cd, Pb, Cu, and Al (Li *et al*., 2006; Hu *et al*., 2007; Da Mota *et al.*, 2008; Siripornadulsil and Siripornadulsil, 2013). In addition, high performance liquid chromatography (HPLC) and liquid chromatography-mass spectroscopy (LC-MS) have been applied to analyze organic compounds of root exudates (Bylund *et al*., 2007; Dundek *et al*., 2011; Jaitz *et al*., 2011). The low-molecular-weight (LMW) compounds, such as organic acids (oxalic, tartaric, malic and succinic acids) and amino acids (proline, threonine, glutamic acid and aspartic acid, etc.), were found in root exudates of plants grown on contaminated soil (Ahonen-Jonnarth *et al*., 2000; Xu *et al*., 2007).

Therefore, the aims of this research study were to evaluate the correlation between rhizobacterial communities, abiotic factors, and biotic factors in bulk soil and rhizospheric soil during maize growth in heavy metal (Zn, Cd, Pb, and Fe) the contaminated area of Pha Te Village, Mae Sot District. Metal accumulation in the phytomass, pollution index, and potential ecological risk index in the field-grown maize were elucidated for sustainable development in the field-grown maize in Mae Sot. A one month pot experiment was undertaken to focus on the impact of heavy metals on maize root extracts relating to plant growth. The results obtained from both field and pot experiments could support the agricultural ecosystems and environment sustainability by phytomanagement and phytoremediation in the heavy metals contaminated area.

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1.2 Objectives

1. Study the correlation between rhizobacterial communities, abiotic factors (rainfall, temperature, pH, Ec, Om, N, P, K, CEC, and soil moisture), and biotic factors (maize growth and metals accumulation) during maize growth in different levels of heavy metals (Zn, Cd, Pb, and Fe) contaminated in maize fields of Pha Te Village, Mae Sot District.
2. Study culturable and unculurable rhizobacteria obtained from each maize growth stage and their identify by 16S rDNA and PCR-DGGE techniques.
3. Study the accumulation of the heavy metals in phytomass (stalk, leaves, male flower (tassel), baby corn, corncob, and seed). Pollution index and potential ecological risk index in field-grown maize were elucidated for sustainable development.
4. Study root extracts obtained from maize grown in various concentrations of the heavy metals by focusing on total phenolic content (TPC), total flavonoid content (TFC), phenolic compounds, and organic compounds.

1.3 Advantages of the study

1. The results of correlations between rhizobacterial communities and abiotic and biotic factors during maize grown in heavy metals (Zn, Cd, Pb, and Fe) can be applied for phytomanagement.
2. The results of culturable and unculturable bacteria isolated from rhizospheric soil of maize grown in heavy metals contamination could specific and imply their heavy metal tolerance properties. In addition, the culturable rhizobacterial isolates should be studied further for their application in maize growth promotion.
3. The results of heavy metals accumulated in phytomass (stalk, leaves, male flower, baby corn, seed, and corncob) can indicate the amount of heavy metals in edible parts and biomass, which can support phytomanagement and phytoremediation.
4. The results of HPLC and LC/MS-MS could specify major phenolic compounds and organic compounds in root extracts of maize grown under the stress of heavy metals, and the compounds might relate to microbial communities in the rhizosphere.

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1.4 Scope of research work

The field site was located in an agricultural area near Mae Tao Creek in Ban Pha Te Village, Phatat Phadaeng Sub-district, Mae Sot District, Tak Province, Thailand (N 16º 40’26” E 98º 37’46”). Soil properties were analyzed by standard methods. Growth of maize was investigated at four stages of V10-tenth-leaf, R1-silking, R4-dough, and R6-maturity. Rhizobacterial communities were studied with both culturable and unculturable techniques for morphological classification including bacterial counts, 16S rDNA, and PCR-DGGE techniques. Plant growth was recorded as height and dry weight. Metals accumulated in the phytomass (stalk, leaves, male flower (tassel), baby corn, seed, and corncob) of maize were digested and analyzed by atomic absorption spectroscopy (AAS) and inductively coupled plasma optical emission spectrometry (ICP-OES). For the pot experiment, root extracts obtained from one month maize grown under three stress levels of heavy metals (Zn, Cd, Pb, and Fe) contaminated soil were examined for TPC and TFC as well as being studied for phenolic compounds and organic compounds by HPLC and LC-MS/MS.

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CHAPTER 2

LITERATURE REVIEW

2.1 Maize *(Zea mays)*

Maize *(Zea mays)* is one of the five major crops grown in the uplands of Thailand, along with rice, cassava, sugar cane, and rubber trees (Ekasingh *et al.*, 2004). Maize is a tall annual plant belonging to the grass family (Gramineae), and it has a fibrous root system and an erect stalk with a single leaf at each node and leaves in two opposite ranks (Figure 2.1) (Benson and Pearce, 1994). The maize plant is often 2.5 m in height. Maize belong to:

Order Poales
Family Poaceae
Subfamily Panicoideae
Tribe Andropogoneae
Genus *Zea*
Species *Z. mays*
Subspecies *Z.mays* subp. mays

(National Plant Germplasm System, 2015)

2.1.1 Growth and development of maize

The growth and development of maize are complex processes. The most common system used for defining maize growth stages is divided into two parts: vegetative growth (V-stage) and reproductive development (R-stage) (Figure 2.2) (NSW, 2009).

Vegetative growth stages are determined by the number of visible leaf collars present (Figure 2.3a). The final V-stage is VT. This is when all the branches of the tassel are fully emerged (Figure 2.3b). Reproductive stages are defined as silking - the emergence of silks beyond the tip of the ear husk (Figure 2.3b). The rest of the R-stages relate to the development of the kernels on the ear (NSW, 2009).

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Figure 2.1 Structure of maize (NSW, 2009)

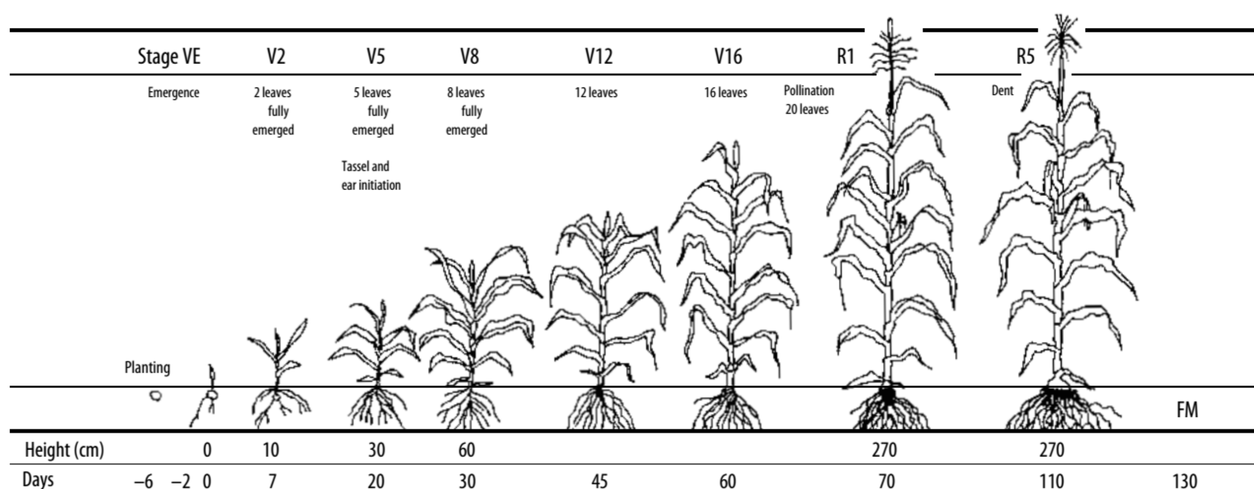

Figure 2.2 Maize growth stages (NSW, 2009)

(a)

(b)

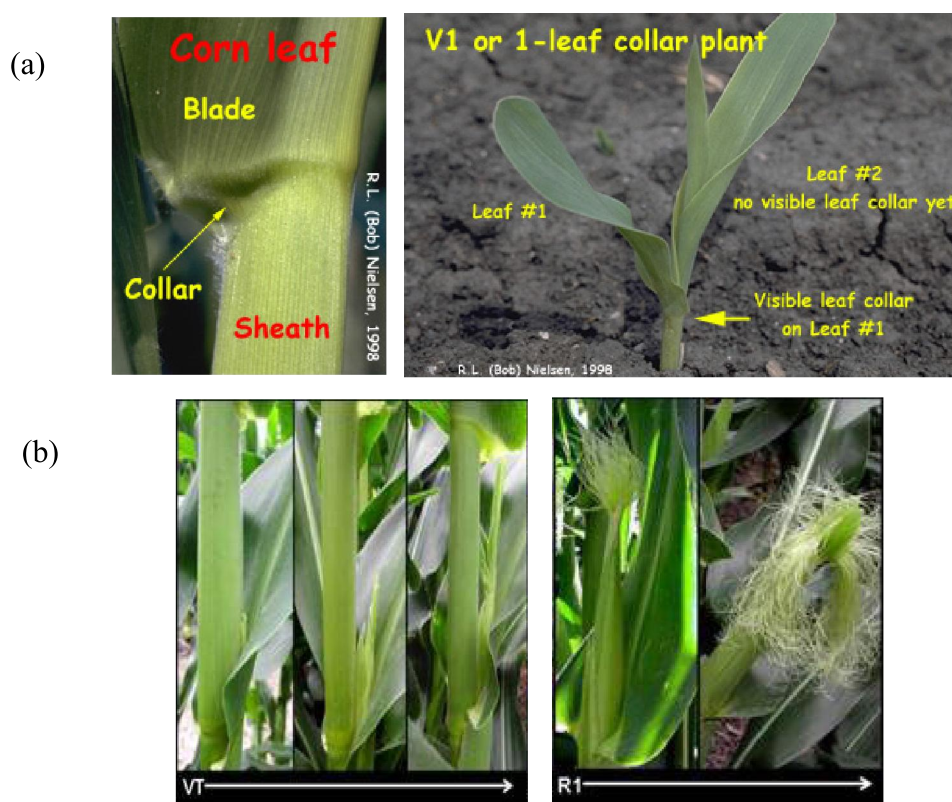

Figure 2.3 Maize collar and maize development form VT to R1. (a) leaf collar and (b) final vegetative stage (VT) and the beginning of the reproductive stage (R1) (Neilson, 2001; http://www.agronext.iastate.edu/corn/)

2.1.2 Trends of maize production

Thai farmers usually plant maize as their major farm enterprise. In addition, rapid economic growth and acceleration of urbanization are expected to create an even higher demand for maize in Thailand. This trend will lead to the intensification of current maize production systems. Therefore, the government promoted crop diversification, increased population growth, improved transportation networks, international trade, and expanded upland farming areas. Increase demand for grains from the domestic livestock and poultry industry stimulated Thailand’s maize production beginning in the 1980s (Figure 2.4) (Ekasingh *et al.*, 2004).

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Figure 2.4 Maize area and production, Thailand, 1961-2011 (http://en.actualitix.com/country/tha/thailand-maize-production.php)

2.2 Zn and Cd toxicity

2.2.1 Toxicity of Zn to humans

The mineral Zn is present in every part of the body and has a wide range of functions. It helps the healing of wounds and is a vital component of many enzyme reactions. Zn is vital for the healthy working of many of the body's systems. It is particularly important for healthy skin and is essential for a healthy immune system and resistance to infection (Fosmire, 1990). However, exposure to high doses has toxic effects, but acute Zn intoxication is a rare event (Plum *et al.*, 2010). Zn will interfere with the metabolism of other minerals in the body, such as iron and copper (Fosmire, 1990; Plum *et al.*, 2010). In addition, Zn influences apoptosis by acting on several molecular regulators of programmable cell death, including caspases and proteins from the *Bcl* and *Bax* families (Plum *et al.*, 2010). Symptoms of Zn toxicity occur after ingestion of one or more grams (Fosmire, 1990; Plum *et al.*, 2010). The presenting symptoms include nausea, vomiting, epigastria pain, abdominal cramps, and diarrhea (frequently bloody) (Fosmire,

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1990) On the other hand, Zn deficiency impacts on growth, neuronal development, immunity, and in severe cases its consequences are lethal (Figure 2.5) (Plum *et al.*, 2010).

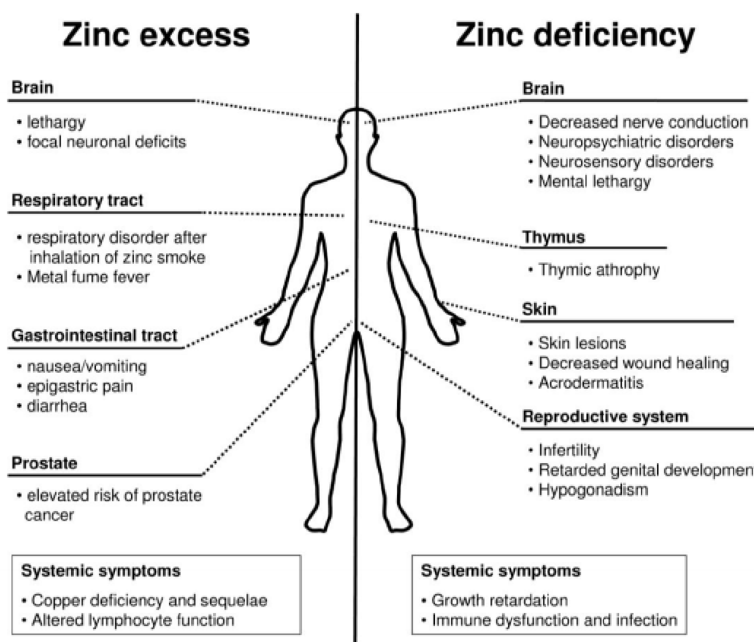

Figure 2.5 Toxicity of Zn to humans (Plum *et al.*, 2010)

2.2.2 Toxicity of Zn to plants

Zn has an essential role in plant metabolic processes, which is significant activity as a component of a variety of enzymes (Kabata-Pendias, 2011). In general, Zn concentrations in plants are between 15-150 ppm (Hagemeyer, 2004). Broadley *et al.,* (2007) reviewed Zn toxicity symptoms in plants, which are usually visible at [Zn] leaf > 300 mg kg^{-1} of leaf dry wt. In the presence of high Zn concentrations, Zn sensitive monocots and dicots have been shown to have a Fe-deficiency, which induced chlorosis through reductions in chlorophyll synthesis and chloroplast degradation (Broadley *et al.*, 2007; Chaney, 1993). Zn deficiency caused low crop production (Hagemeyer, 2004). For example, corn showed white to yellow bands beginning at the base of the leaf, but the midrib and leaf margins remain green (Idaho, 2009) (Figure 2.6).

Figure 2.6 Zn deficiency in maize (Idaho, 2009)

2.2.3 Toxicity of Cd to humans

Cd is absorbed more efficiently by the lungs (30 to 60%) than by the gastrointestinal tract, with the latter being a saturable process (Nordberg *et al.*, 1985). It is transported in the blood and widely distributed in the body but accumulated primarily in the liver and kidneys (Kilcup, 2013). Moreover, a Cd burden (especially in the kidneys and liver) tends to increase in a linear fashion up to about 50 or 60 years of age after which the body burden remains somewhat constant. Metabolic transformations of cadmium are limited to its binding to protein and nonprotein sulfhydryl groups, and various macromolecules, such as metallothionein, which is important in the kidneys and liver (ATSDR, 1989). Cd is excreted primarily in the urine. For example, the inhabitants in Mae Sot District, Tak Province, Northwestern Thailand, who have had health impacts of Cd overexposure (Swaddiwudhipong *et al.*, 2007; 2010), such as risk of renal dysfunction and chronic diseases (Teeyakasem *et al.*, 2007; Limpatanachote *et al.*, 2009). In addition, acute oral exposure to 20-30 g has caused fatalities in humans (Young, 1991).

2.2.4 Toxicity of Cd to plants

Cd is a non-essential and highly toxic metal. Plant Cd concentrations are between 0.05-0.2 ppm, but can be much higher on contaminated sites (Hagemeyer, 2004). High Cd levels decrease the dry weight of plants and cause plant death because Cd toxicity resulted in growth retardation, inhibition of photosynthesis, inhibition of enzymes, and generation of free radicals (Prasad, 1995; Das *et al.*, 1997). In general, overt symptoms induced by elevated Cd contents in plant are chlorosis of leaves and red-brown

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coloration of leaf margins or veins (Figure 2.7) (Kabata-Pendias, 2011). In addition, Cd toxicity drastically reduced plant growth (plant dry mass and leaf area), photosynthetic traits (net photosynthetic rate, stomatal conductance, and internal CO_2 concentration), and the contents of ascorbic acid (AsA), glutathione (GSH) and potassium (K) (Umar *et al.*, 2008). Prasad (1995) reported cereal plants, such as maize (*Zea mays*), oat (*Avena sativa*), barley (*Hordeum vulgare*) and rice (*oryza stiva*), are sensitive to Cd toxicity.

Figure 2.7 Cadmium (Cd) toxicity symptoms, i.e., necrosis appeared in older mustard leaves that led to defoliation (Umar *et al.*, 2008)

2.3 Rhizospheric process

2.3.1 Roles of rhizosphere

The rhizosphere is the zone of soil surrounding a plant root where the biology and chemistry of the soil are influenced by the root (Wenzel *et al.*, 2004). This area has intense biological and chemical activity influenced by compounds exuded by the root (root exudates) and by microorganisms feeding on the compounds (Lines-Kelly, 2005; Kowalchuk *et al.*, 2010; Berendsen *et al.*, 2012). Figure 2.8 shows the interactions in the rhizosphere by plants that are able to influence the composition and activation of their rhizosphere microbiome through exudation of compounds that stimulate (green arrows) or inhibit (red blocked arrows) (Berendsen *et al.*, 2012). Which affect every other organism to a certain extent through a complex network of interactions. The diversity, abundance, and activity of bacterial communities in the rhizosphere depended on many factors, such as the physico-chemical and structural characteristics of the soil (Griffiths *et al*., 2003). Sarathchandra *et al.*, (1997) showed that the bacterial communities of white

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clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.) with 0-5 cm soil depths were significantly higher than with those with 5-10 cm soil depths. On the other hand, physical model system investigating interactions between microorganisms isolated from the rhizosphere showed the distribution of bacteria did not change significantly as depth increased (Pearce *et al.*, 1997).

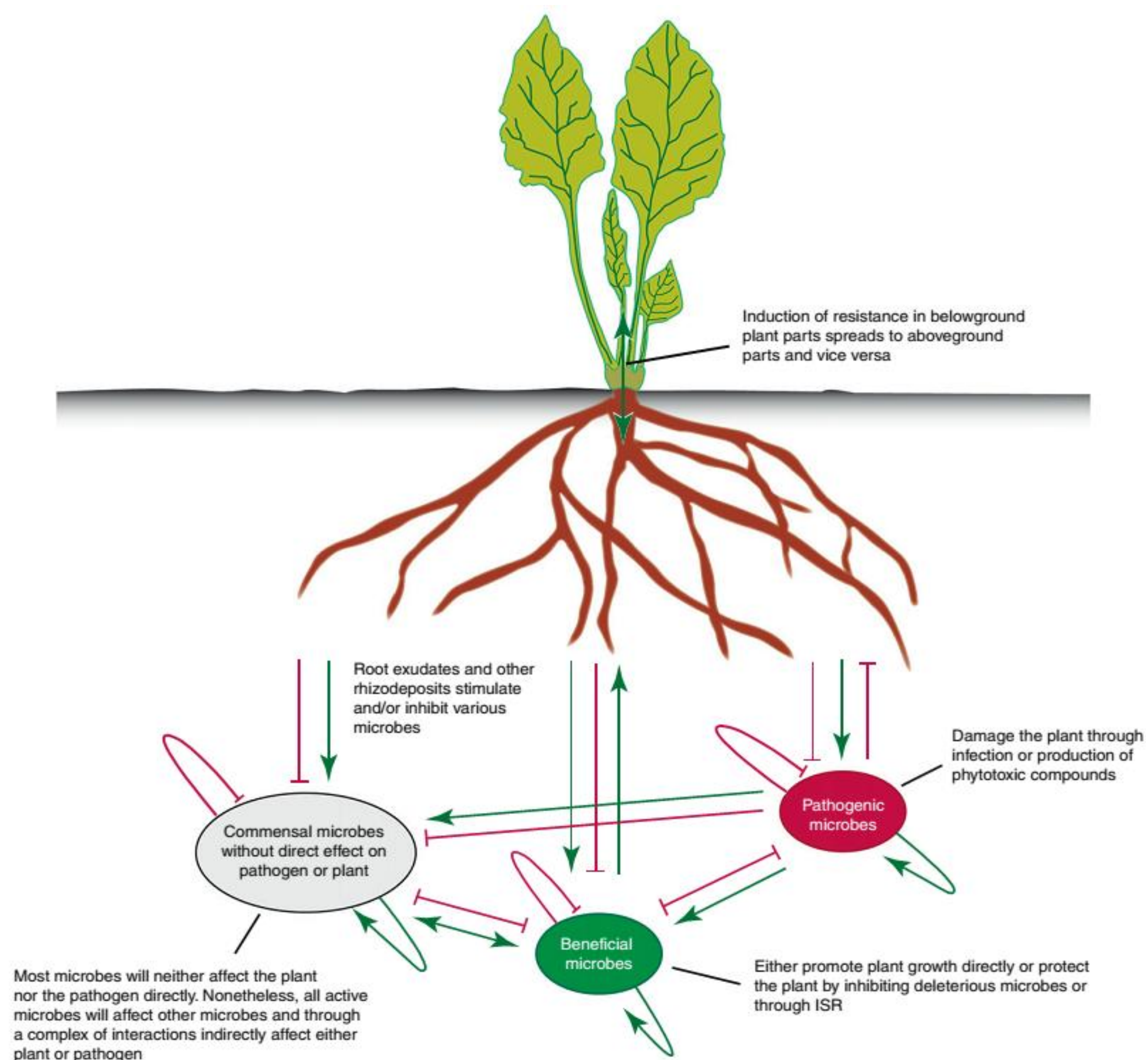

Figure 2.8 Interaction rhizosphere (Berendsen *et al.*, 2012)

The soil microbial population can be measured by comparing the population density (colony forming units, CFU) between the rhizospheric (R) and bulk soil (S), in terms of the “R/S ratio”. In addition, the differences in their colony morphologies were shown in terms of color, shape, and size, etc. (Hu *et al.*, 2007). The effect of

microbiological activity in the rhizospheric soil is much greater than in the bulk soil from plant roots (Table 2.1). Osorio Vega (2007) reported the rhizosphere effect was higher in bacteria than fungi.

Table 2.1 Number of bacteria (CFUx10^6 g^{-1} soil or root dry wt.) in rhizospheric (R) and bulk soils (S)

Plant species	Rhizosphere (R)	Bulk (S)	R/S ratio
Maize (*Zea mays*)	4,500	184	3
Oats (*Avena sativa*)	3,588	184	6
Wheat (*Triticum aestivum*)	4,119	120	6
Barley (*Hordeum vulgare*)	3,216	140	3

Modified form Osorio Vega (2007)

2.3.2 Roles of root exudates

Root exudates are the substrates released by the roots. They are involved in important functions of the rhizosphere bacterial community (Rovira, 1969) and providing defense against pathogenic microorganisms (Manoharachary and Mukerji, 2006). Root exudates are often divided into two classes of compounds. Low molecular weight compounds, such as amino acids, organic acids, sugars, phenolics, and other secondary metabolites, which form the high diversity of root exudates. High molecular weight exudates, such as mucilage (polysaccharides) and proteins, are less diverse but often compose a larger proportion of the root exudates by mass (Walker *et al.*, 2003; Bais *et al.*, 2006; Narula *et al.*, 2009). The classes of compounds released in plant root exudates and functions are summarized in Table 2.2. However, a systematic study to determine the complexity and chemical composition of root exudates from diverse plant species has not been undertaken (Walker *et al.*, 2003).

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Table 2.2 Classes of compounds released in plant root exudates

Classes of compounds	Components	Functions
Aliphatic acid	Formic, acetic, butyric, propionic, maleic, malic, citric, isocitric, oxalic, fumaric, malonic, succinic, tartaric, oxaloacetic, pyruvic, oxaloglutaric, glycolic, shikimic, acetonic, valeric, gluconic, and quini	Plant growth regulation, chemoattractants, and microbial growth stimulation
Aromatic acids	p-hydroxybenzoic, caffeic, pcoumeric, ferulic, gallic, gentisic, protocatechuic, salicylic, sinapic, and syringic	Plant growth regulation and chemoattractants
Phenolics	Flavanol, flavones, acetosyringone, flavanones, anthocyanins, and isoflavonoids	Plant growth regulation, allelopathic interactions, plant defence, hytoalexins, chemoattractants, initiate legumerhizobia, arbuscular mycorrhizal and actinorhizal interactions, microbial growth stimulation, and stimulate bacterial xenobiotic degradation
Hormones	Auxin, ethylene and its precursor 1-aminocyclopropane-1-carboxylic acid, putrescine, jasmonate, and salicylic acid	Plant growth regulation

* Adapted from Bertin *et al.,* 2003; Somers *et al.,* 2004; Neumann and Römheld, 2007; Badri, 2009; Shi, 2009

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The sampling of root exudates still remains a very challenging task. The difficulties of accessing the rhizosphere without disturbance or damage to the plant roots and selecting a suitable collection medium without affecting the root physiology and exudate recovery are common problems for the study of root exudates (Rovira, 1968; Neumann and Römheld, 2007; Phillips *et al.*, 2008). A summary of methods commonly used for root exudate sampling based on solution culture approaches and nutrient solution-grown plants, are given in Table 2.3 (Oburger *et al.*, 2013).

Root exudate analysis has been used successfully for the identification of a wide range of different plant metabolites. Root exudates can be detected by gas chromatography mass spectrometry (GC-MS) or GC-MS coupled with other techniques (e.g., nuclear magnetic resonance) (Dundek *et al*., 2011), high performance liquid chromatography (HPLC) (Collins, 2004), and reversed-phase column liquid chromatography (RPLC) (Cawthray, 2003). However, exudate sampling usually has extremely low concentrations of exudate compounds, and thus the analysis requires sample preparation (e.g., pre-concentration) (Neumann *et al.*, 2009; Dundek *et al*., 2011). In addition, the amount of carbon is another way to detection exudation released in soil; it is usually determined by a wet digestion method and analyzer (Dundek *et al.*, 2011).

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Table 2.3 Advantages and disadvantages of techniques frequently used for root exudate sampling

Plant growth	Exudate sampling	Advantages	Disadvantages
Nutrient solution (hydroponic)-aerated	Trap solutions: H_2O, $CaCl_2$, and $CaSO_4$	-No adsorption of exudates to soil particles. -Microbial degradation can be inhibited.	-Large sampling volume. -Different nutrient availability and O_2/CO_2 status.
Soil: pots or rhizotrons	Soil washed out and roots transferred to trap solutions: H_2O, $CaCl_2$, and $CaSO_4$	-No adsorption of exudates to soil particles. -Microbial degradation can be inhibited.	-Root damage and sudden change in environment can alter exudation rates. - Large sampling volume.
Soil/sand/ vermiculite columns	Leaching of planted column	- (Semi) natural growth condition. -Natural root proliferation. -Non-destructive.	-Exudate concentration potentially altered by adsorption processes and microbial degradation as well as exudation. -Large sampling volume with low exudate concentration.
Soil: rhizotrons, rhizoboxes	Rhizosphere soil solution: micro-suction-cups	-Determination of rhizosphere concentration gradients. -Single point sampling.	-Exudate concentration altered by adsorption processes and microbial degradation as well as exudation. -Very small sampling volume.

Modified form Oburger *et al.*, 2013

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2.3.3 Beneficial effects of rhizosphere microbes on soil and plants

Soil microbes are very important in biogeochemical cycles and have beneficial effects on plant growth by providing nutrients and growth factors or by producing antibiotics and siderophores (Hayat *et al.*, 2010). These are associated with the rhizosphere, such as plant growth promoting rhizobacteria (PGPB) and mycorrhizal and sulphate reducing bacteria (Figure 2.9). On the other hand, these inhibit plant growth by secreting phytotoxins.

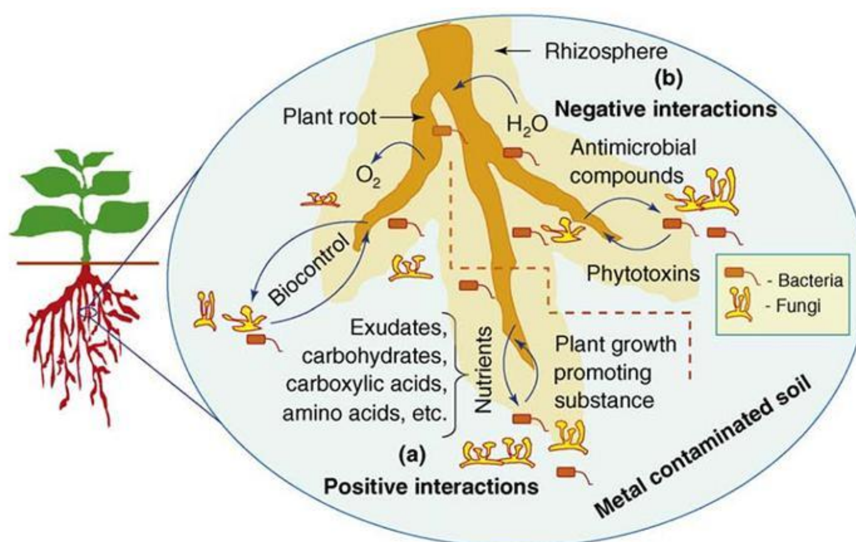

Figure 2.9 Interaction between plants and microbes in rhizosphere can be classified as either (a) positive or (b) negative interactions (Rajkumar *et al*., 2009)

Free-living soil bacteria beneficial to plant growth are usually referred to as plant growth promoting rhizobacteria (PGPR) (Hayat *et al.*, 2010). They have the ability to promote plant growth by various mechanisms, including synthesis of phytohormones, such as indole-3-acetic acid (IAA), nitrogen fixation, phosphate solubilisation, and aminocyclopropane-1-carboxylate (ACC) deaminase (Osorio Vega, 2007; Hayat *et al.*, 2010; Kumar *et al.,* 2011). PGPB can protect the host plants from pathogens by antibiotic, siderophore, and extracellular enzyme production (Mukerji *et al.*, 2006). In addition, PGPB can oxidized soil manganese (Mn) in term of Mn^{4+}, in the low-soluble mineral Pryolusite (Osorio Vega, 2007).

A mycorrhiza is a symbiotic relationship between a fungus and the root of a vascular plant. It has been classified and grouped together based on the colonization of the host plant root, either intracellular as in arbuscular mycorrhizal fungi (AMF or AM) (Figure 2.10a) or extracellularly as in ectomycorhizal fungi (Figure 2.10b) (Linderman, 1988; Badri *et al.*, 2009). Mycorrhizal associations are present in almost all land plants and are essential biological constituents of the rhizosphere (Badri *et al.*, 2009). In soils, which are deficient or have less available minerals, mycorrhizal fungi increase the efficiency of mineral uptake, such as calcium (Ca), iron (Fe), magnesium (Mg), copper (Cu), potassium (K), nitrogen (N), and zinc (Zn) (Linderman, 1988; Habte, 2000; Osorio Vega, 2007; Ortas, 2010). Mycorrhizal can also benefit plants by the production of substances affecting plant growth, increasing photosynthesis, improving osmotic adjustment under drought and salinity stresses, and protection against root pathogens, pests, and soil borne diseases (Linderman, 1988; Timonen and Marschner, 2006; Osorio Vega, 2007; Ortas, 2010).

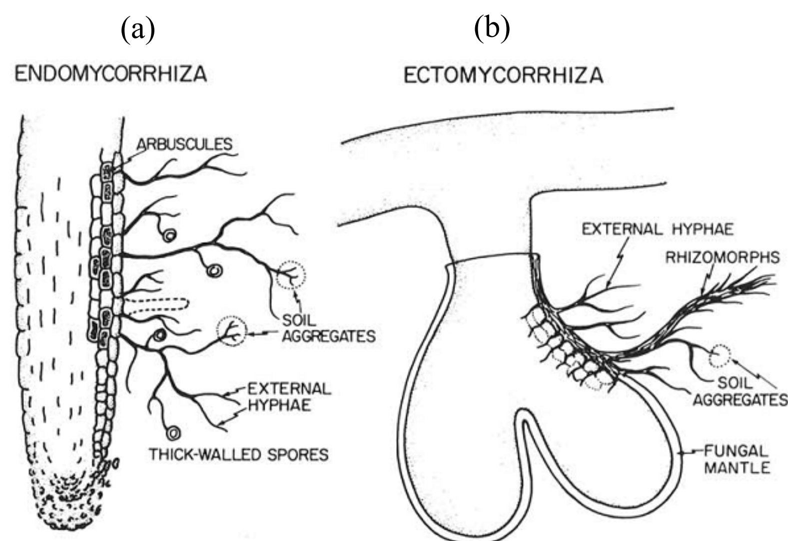

Figure 2.10 Morphological changes in roots as they become mycorrhizal and effects of those changes on the development of a mycorrhizosphere: (a) endomycorrhiza and (b) ectomycorrhizal (Linderman, 1988)

Microorganisms that play an important part in sulphur transformations are sulphate-reducing bacteria (SRB). There are bacteria and archaea that can obtain energy

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by terminal electron acceptors for the degradation of organic compounds, resulting in the production of sulphide (Barton and Tomei, 1995; Muyzer and Stamp, 2008). The use of SRB may have a positive ecological effect on bioremediation and plant growth, i.e., initiation of active elongation (vegetative growth) and commencement of reproduction and affect sulphate reduction in sediments inhabited (Hines *et al.*, 1999; Jiang and Fan, 2008). However, SRB can cause a serious problem for industries, such as the offshore oil industry, because of the production of sulphide, which is highly reactive, corrosive, and toxic (Muyzer and Stamp, 2008).

2.4 Methods and techniques to study rhizosphere microbes

2.4.1 Soil sampling and storage

The rhizospheric soil generally is a microcosm inhibited by a wide range of soil microorganisms. Sampling of soils is crucial for assessing microbiological soil parameters, including the functioning of the soil ecosystem, which is related to the presence of specific microbial groups (Van Elsas *et al.*, 2002). Most microbiological, biochemical, and soil chemical assays require a small sample size (approximately 100 g of soil) to a medium sample size (100 g to several kilograms of soil), whereas a large sample size (over several kilograms) may be needed when undisturbed soil cores are to be used or for certain soil industrial application (Van Elsas *et al.*, 2002). The small to medium sized samples can be collect form the soil horizon using a hand auger, sample corer, spade, shovel, or trowel, whereas a large sample size requires specialized methodology, such as drilling, boring, or preparation of trial pits (Van Elsas *et al.*, 2002). The field samples should be preserved in such a way that characteristics are not lost or changed (Anderson, 1987). The top soil layers are approximately 15 to 20ºC, which may include keeping the sample cool (4ºC or on ice). However, samples should be quickly stored at least at -20ºC when sample DNA is the target (Van Elsas *et al.*, 2002).

2.4.2 Microbial separation from rhizospheric soil

The rhizospheric soil has been defined as a thin layer of soil adhering to the root. Many methods have been used to separate rhizospheric microbes from soil. Table 2.4 shows methods for the removal of rhizosphere microorganisms from plants, such as

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Table 2.4 Methods for removal of rhizosphere microorganisms from plant

Method	Advantages	Disadvantages
Root washing	- Numerous samples can be processed at once. - Time consumed is 30 min to several hours.	- Does not remove tenacious surface microbes. - Liquid may promote microbial growth.
Vortexing	- Can remove tenacious microbes from root surface. - Sample processed rapidly (30 s to 2 min).	- Usually must be processed individually.
Homogenizer	- Effective at removing tenacious microbes from surface.	- Only one sample may be processed at a time. - Sample may contain endophytic microbes.
Blender or trituration	- Effective at removing tenacious microbes from surface. - Equipment relatively inexpensive. - Sample processed rapidly.	- Only one sample may be processed at a time. - Sample may contain endophytic microbes.
Ultrasonication		
1. Root imprints on agar	- Visualization of the in situ spatial patterns of bacteria.	
2. Adhesive removal	- Remove fungal spores, hyphae, some bacterial cells. - Retains spatial information and can be viewed microscopically. - Inexpensive method.	- Serial dilution not possible. - Difficult to culture removed microbes.

Modified form Dandurand and Knudsen, 2002

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root washing technique, vortexing, sonication, and blending or maceration (Dandurand and Knudsen, 2002). However, the method can affect the recovery of the populations (Donegan *et al.*, 1991; Kloepper *et al.*, 1991; Dandurand and Knudsen, 2002).

2.4.3 Isolation techniques in microbiology

Two general approaches to quantitative estimation of the microbial population in the rhizosphere are direct methods, such as visualization techniques, and indirect methods, such as dilution plating (Cochran, 1950; Dandurand and Knudsen, 2002).

Visualization techniques take advantage of direct observation of microorganisms on the rhizosphere (Campbell and Rovira, 1973), determination of metabolically active bacteria and fungi (Bottomley, 1994), and application in microbial ecology (Bohlool and Schmidt, 1980). On the other hand, isolation and purifying nucleic acids from environmental samples have been devised, and they employ some of the same basic techniques described in molecular biology (Dandurand and Knudsen, 2002).

2.4.4 Enumeration methods for rhizospheric microbes

A variety of methods can be used to investigate the microbial populations in the rhizosphere. Finding a suitable method for a study is a challenging task because some organism numbers may be too large or some too small. Therefore, the methods for the enumeration of microorganism have evolved (Hurst *et al.*, 1997), which involves dilution, concentration, or enrichment of populations to examine the numbers (Figure 2.11) (Ranganayaki *et al.*, 2006).

Most DNA-based approaches are for predicting the identities of microbial communities, which has become the dominant signature molecule for community analysis. The molecular analysis of cultivated samples enables increased analyses of the links between phylogenies created from rRNA genes and functional genes, thus facilitating identification of microorganisms performing particular ecosystem functions, such as characterizing the structure, activity, and function of complex microbial communities (Prosser *et al.*, 2010). The commonly used genetic fingerprinting techniques are polymerase chain reaction (PCR) dependent approaches and include denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (T-RFLP), single strand

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conformational polymorphism (SSCP), cloning-sequencing, and pyrosequencing (Pyrotags) (Shi, 2009; Prosser *et al.*, 2010).

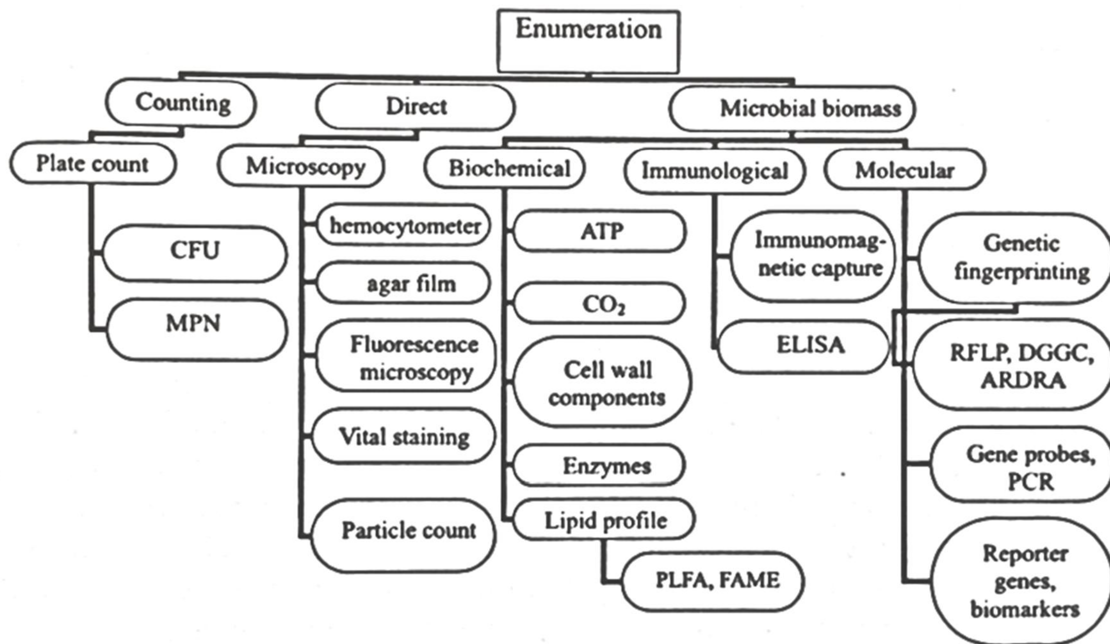

Figure 2.11 Conventional and modern enumeration methods (Ranganayaki *et al.*, 2006)

However, the relative abundance of phylogenetic groups is inferred from, e.g., the relative abundances of phylotypes within clone libraries, relative intensities of DGGE bands, and relative peak areas in T-RFLP electropherograms, which have a problem inherent that DGGE bands or T-RFLP peaks may contain more than one sequence (Prosser *et al.*, 2010). The advantages and disadvantages of some genetic fingerprinting techniques are presented in Table. 2.5.

Table 2.5 Advantages and disadvantages of genetic fingerprinting techniques

Fingerprinting approach	Key feature	Advantages	Disadvantages
DGGE/TGGE	Separation of PCR products based on their melting or temperature denaturation gradient.	- Bands of interest can be cloned and sequenced to identify corresponding populations.	- Only the most dominant members of the community are detected. - One band can represent several sequences.
T-RFLP	Separation of PCR products based on the size of their fluorescent labels.	- Suitable for comparing large number of samples over time or according to treatment.	- Only putative identities of populations corresponding to a given TRF can be obtained by comparison to sequences deposited in databases that are digested in silico. This problem is reduced by using multiple restriction enzymes in parallel.
Clone libraries	Sequence analysis of clones following PCR amplification.	- Provides information for phylogeny and identification.	- Many clones need to be sequenced to obtain the required coverage of diversity.
454 pyrosequencing	Pyrosequencing of PCR products attached to beads in a Pico TiterPlate.	- Very large amounts of sequences (>100,000) can be obtained to generate deep coverage of a community.	- Currently limited by availability of 454 FLX sequencers. - The read lengths vary depending on the platform used.

Modified form Prosser *et al.*, 2010

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2.5 Rhizospheric processes in phytoremediation technologies

Phytoremediation is a biological treatment process that utilizes natural processes harbored in (or stimulated by) plants to enhance degradation and removal of contaminants in contaminated soil or groundwater (Alvarez and Illman, 2006). Phytoremediation utilizes physical, chemical, and biological processes to remove, degrade, transform, or stabilize contaminants within soil and groundwater. The success of phytoremediation is strongly determined by the amount of plant biomass present and the concentration of heavy metals in plant tissues. Some plant species, so-called hyperaccumulators (e.g., *Thlaspi goesingense*, *Alyssum bertolonii,* and *Alyssum murale*), which naturally grow in heavy metal contaminated sites, were found to have the ability to accumulate unusually high concentrations of heavy metals without any impact on their growth and development (Lasat, 2002). The interface between microbes and plant roots (rhizosphere) is considered to greatly influence the growth and survival of plants. Therefore, alternative phytoremediation methods that exploit rhizosphere bacteria to reduce metal toxicity to plants have been investigated. Furthermore, the discovery of rhizosphere bacteria that are heavy metal resistant and able to promote plant growth have raised high hopes for ecologically friendly and cost-effective strategies towards reclamation of heavy metal polluted soils. The mechanisms for heavy metal remediation are phytoextraction, phytostabilization, and phytovolatization (Raskin and Ensley, 2000; Prasad, 2011).

- *Phytoextraction:* This application aims to extract and translocate heavy metals, in soluble form, from shallow contaminated soil to plant tissues (Prasad, 2011). It could benefit from rhizosphere processes primarily through plant-microbe induced solubilization prior to uptake by plants, such as the exudation of organic compounds that can decrease the pH and thus solubilize metal cations (Wenzel *et al.*, 2004).

- *Phytostabilization/immobilization:* This application aims to prevent the dispersion of contaminated sediments and soil by using plants (mainly grasses) to minimize erosion by wind or rain action (Prasad, 2011). Hyphae of mycorrhizal fungi and some of root exudates (sugar and mucilage), can mechanically bind together soil particles, making the soil more resistant to erosion (Miller and Jastrow, 1990; Wenzel *et al.*, 2004; Prasad, 2011). In addition, metal cations, oxyanions of metals, and metalloids, may be

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immobilized by the release of exude compounds into the rhizosphere (Wenzel *et al.*, 2004).

- *Phytovalatilization:* The natural ability of a plant to volatilize a contaminant that has been taken up through its roots can be exploited from the leaf stomata or from plant stems (Prasad, 2011; ITRC, 2009). It has been related to specific enzymes produced by rhizospheric microorganisms, which can transform mercury (Hg), selenium (Se), and arsenic (As) to volatile compounds (Terry and Zayed, 1994; Azaizeh *et al.*, 1997; Wenzel *et al.*, 2004).

2.6 Impact of Zn and Cd on rhizospheric process

2.6.1 Impact on microbial community

Many research studies indicated a shift in the diversity of the microbial population after a pollution event with Zn and Cd. Different microbial receptors, such as bacteria, fungi, and actinomycetes, showed a different sensitivity to elevated Zn and Cd levels (Walker, 2008; Margesin *et al.*, 2011). Xin-Xian *et al.,* (2009) showed rhizospheric microbial population associated with hyperaccumulator growing natively on a Pb/Zn mining site, and higher numbers of culturable bacteria, actinomycetes, and fungi were found in the rhizospheric soil than the bulk soil. Cd contamination around the zinc mine had a significantly positive effect on the microbial diversity index (Kositanont, 2009). Walker, (2008) showed a different microbial population between contaminated soil and uncontaminated. However, Zn, Cd, Cu, and Pb contamination decreased both biomass and diversity of the bacterial community in the soil (Li *et al.*, 2006; Hu *et al.*, 2007).

2.6.2 Impact on root exudates

The amounts and compositions of root exudates released by plants are affected by toxic metals (Ma, 2000; Chaffai, 2006; Badri and Vivanco 2009). Heavy metals induce disturbances in biochemical pathways of organic acid and phenolic compound metabolism. Figure 2.12 shows model mechanisms involved in the release of root exudates, such as direct diffusion through the lipid bilayer of the plasmalemma. It depends on the physiological state of the root cell and on the polarity of the exudate compounds facilitating the permeation of lipophilic exudates (Neumann and Römheld, 2007). Mariano *et al.*, (2005) showed aluminum-induced disturbances in biochemical

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pathways of organic acid metabolism by Al that lead to altered concentrations of carboxylic acid in the root. Figure 2.13 shows carbon pathways relevant to the status of particularly malate and citrate, which are both intermediate metabolites of the tricarboxylic acid (TCA) cycle. Malate can be alternatively converted to pyruvate by the action of the malic enzyme, and thereby increase the supply of the substrate for the synthesis of citrate (Taiz and Zeiger, 2002). Changes in the activity of phosphoenolpyuravte carboxylase (PEPC) could have a marked effect on the cell carbon supply and, thus, on organic acid metabolism (Naik and Nicholas, 1986; Ryan *et al.*, 2001). The concentration of organic acids in root exudates of *Pinus sylvestris* significantly increased in soils containing toxic metals, such as Cd (Ahonen-Jonnarth *et al.*, 2000). Xu *et al.,* (2007) reported ryegrass revealed increase low-molecular-weight (LMW) organic acids, such as oxalic, tartaric, malic, and succinic acids, and amino acids, such as proline, threonine, glutamic acid, and aspartic acid, *etc.*, when it grew under soil contamination of 4 mmol Zn kg^{-1}. Zn affected the root exudates, soil pH, and dissolved organic carbon concentrations in the rhizosphere of a hyperaccumulating ecotype compared to the bulk soil (Li *et al.*, 2011).

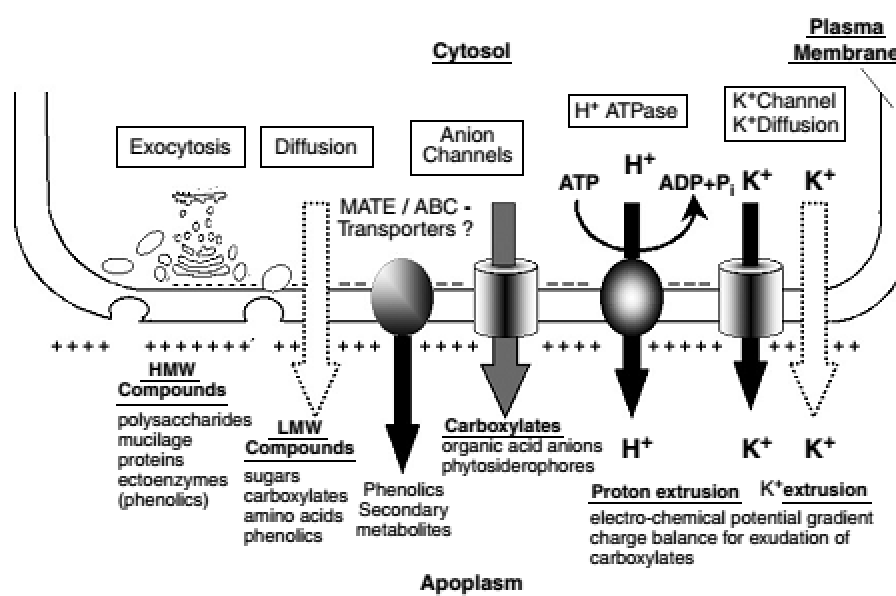

Figure 2.12 Model for mechanisms involved in release of root exudates (Neumann and Römheld, 2007)

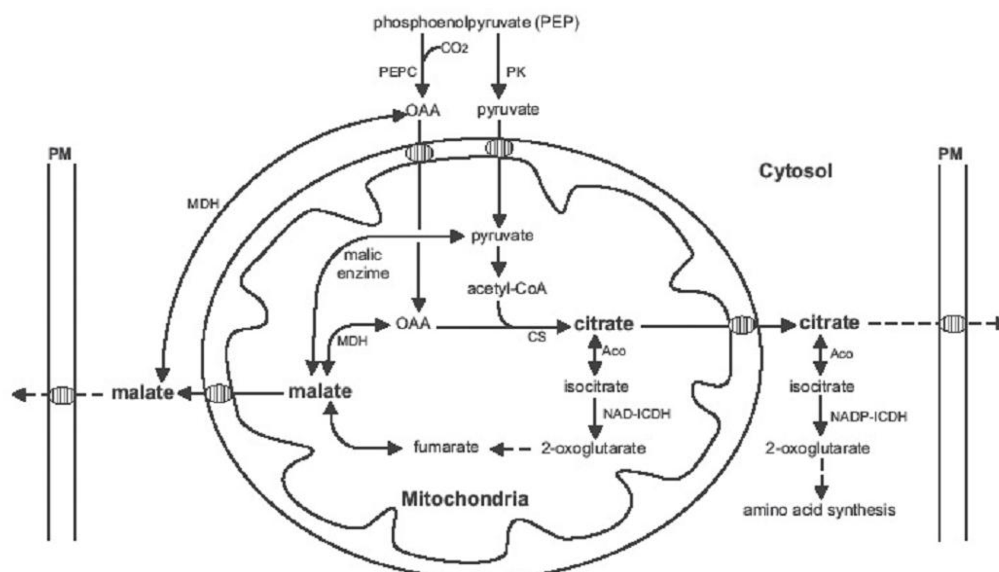

Figure 2.13 Diagrammatic representation of carbon pathways in plant cells related to malate and citrate. Aconitase (Aco), citrate synthase (CS), malate dehydrogenase (MDH), NAD specific isocitrate dehydrogenase (NAD-ICDH), NADP specific isocitrate dehydrogenase (NADP-ICDH), oxaloacetate (OAA), phosphoenolpyruvate (PEPC), pyruvate kinase (PK), plasma membrane (PM). Hatched ellipses on the plasma membrane and mitochondria denote membrane transporters (Mariano *et al.*, 2005).

In addition, the root of many plants exposed to heavy metals exude high levels of phenolics. A complex system consisting of uptake/efflux, transport/sequestration, and chelation is shown in Figure 2.14. The structures of some natural phenolic compounds with high affinity for Al are shown in Figure 2.15. Michalak (2006) explained that heavy metals induce oxidative stress in cells. Wei and Guo (2014) suggested that dietary flavonoids may affect Zn homeostasis, uptake, and transport. Heavy metals (Cd, Pb, Cu, Cr, and Hg) cause oxidative stress, probably through indirect mechanisms, such as interaction with the phenolic compound defense, disruption of the electron transport chain, or induction of lipid peroxidation (Gill, 2014). Plant phenolic compounds can scavenge harmful active oxygen species that are produced under heavy metal exposure or other conditions.

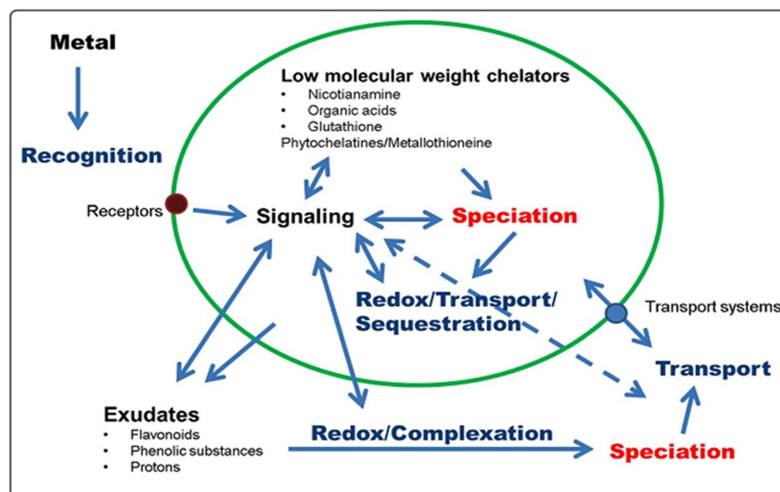

Figure 2.14 Short overview of some important aspects of cellular metal interaction (Viehweger, 2014)

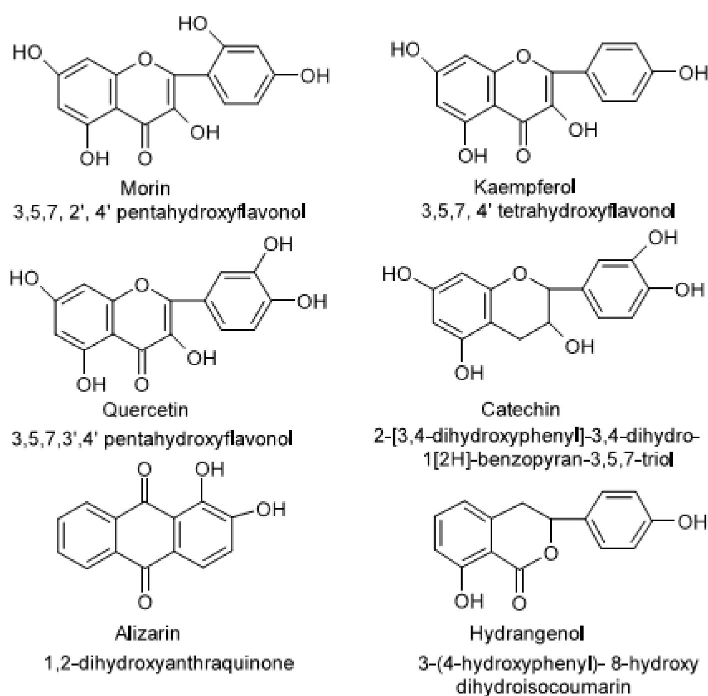

Figure 2.15 Structures of some natural phenolic compounds with high affinity for Al (Barcelo *et al.,* 2002)

CHAPTER 3

METHODOLOGY

This research was designed to investigate the interaction of maize, heavy metals (Zn, Cd, Pb, and Fe) contaminated soil, environmental parameters (rainfall and temperature), abiotic factors (i.e., pH, OM, EC, etc.), and bacterial community in a maize field following conservation practices. The soil and plant samples were collected from five stages of maize growth, which were VE (emerge), V10 (tenth-leaf), R1 (silking), R4 (dough), and R6 (maturity). Communities of culturable and unculturable bacteria were investigated from bulk soil and rhizospheric soil by microbial morphology and population, 16S rDNA, and PCR-DGGE techniques. The amounts of heavy metals accumulation in the phytomass (stalk, leaves, male flower, baby corn, seed, and corncob) were analyzed. Heavy metal contaminations of soils were calculated for the pollution index and potential ecological risk index. The maize growth and root extraction were studied in a pot experiment to obtain the effect of heavy metals (Zn, Cd, and Pb) on root extracts in terms of total phenolic content (TPC), total flavonoid content (TFC), phenolic compounds, and organic acids.

3.1 Research diagram

3.2 Materials and methods

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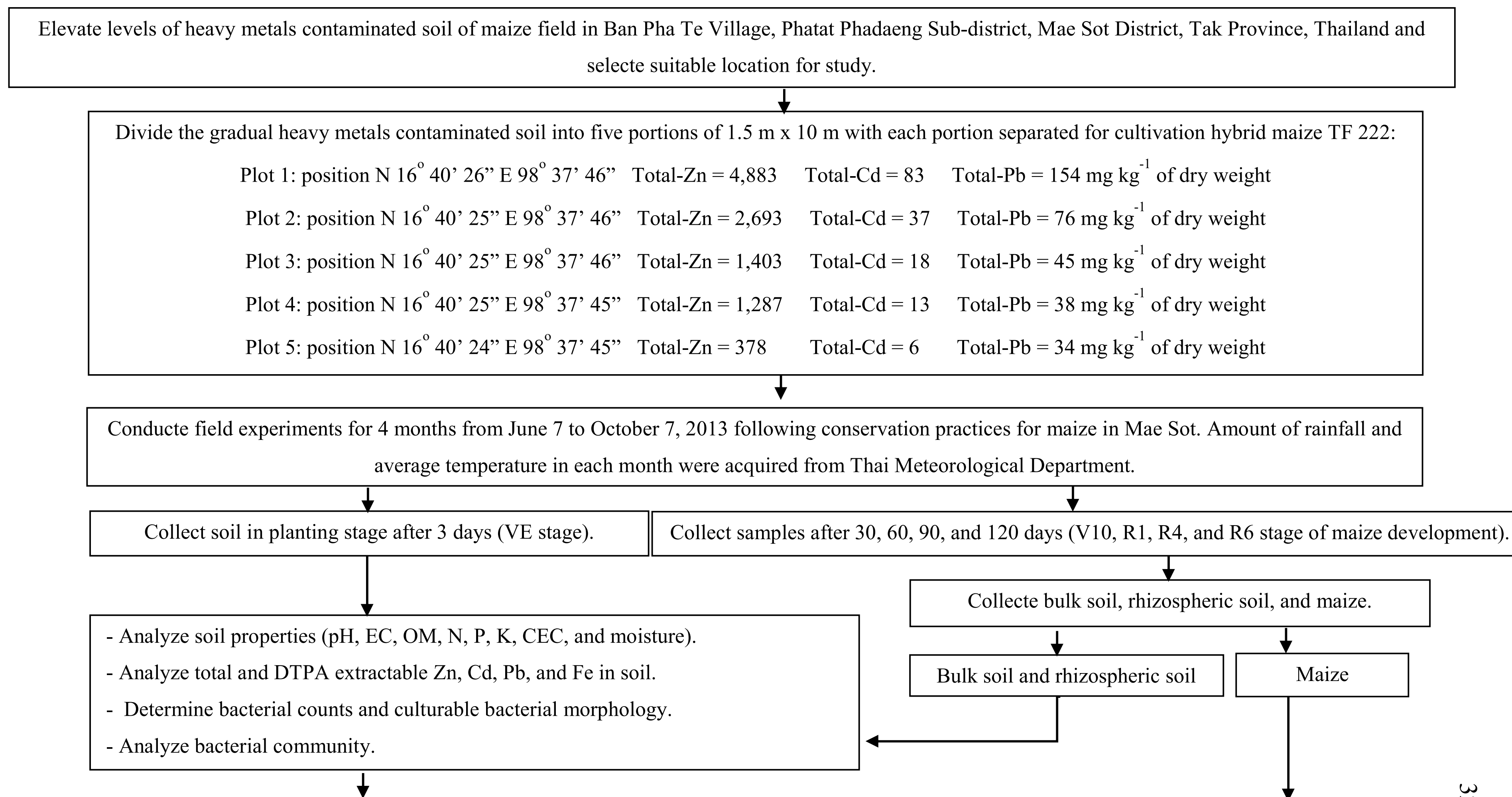
3.1 Research diagram

3.1.1 Field experiments



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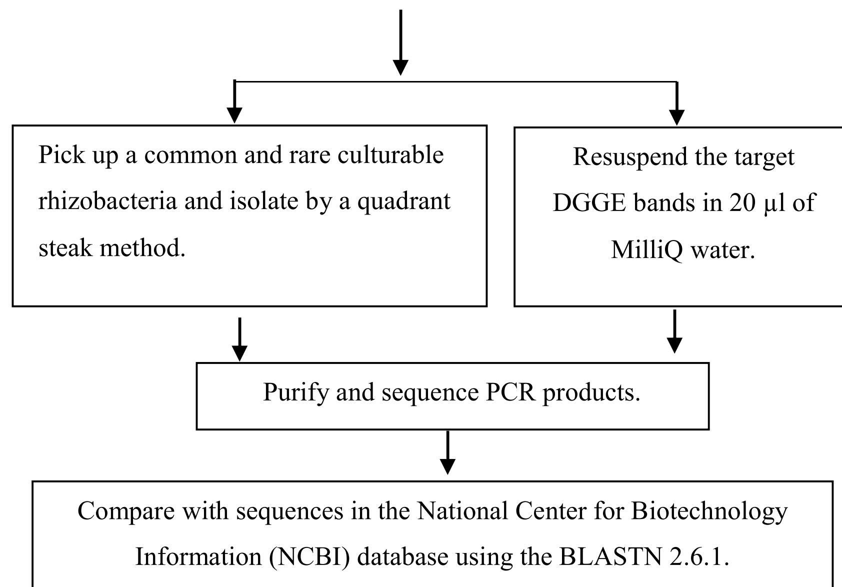

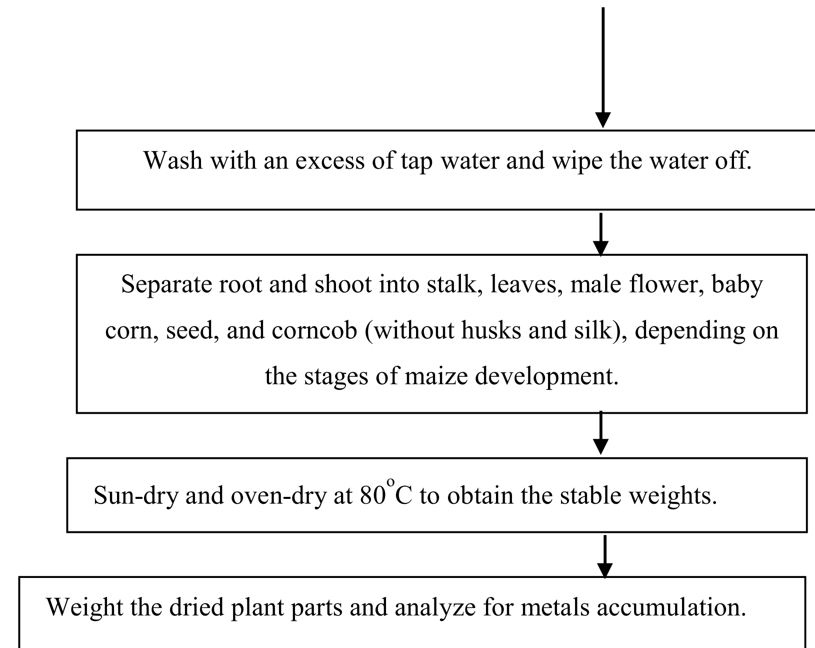

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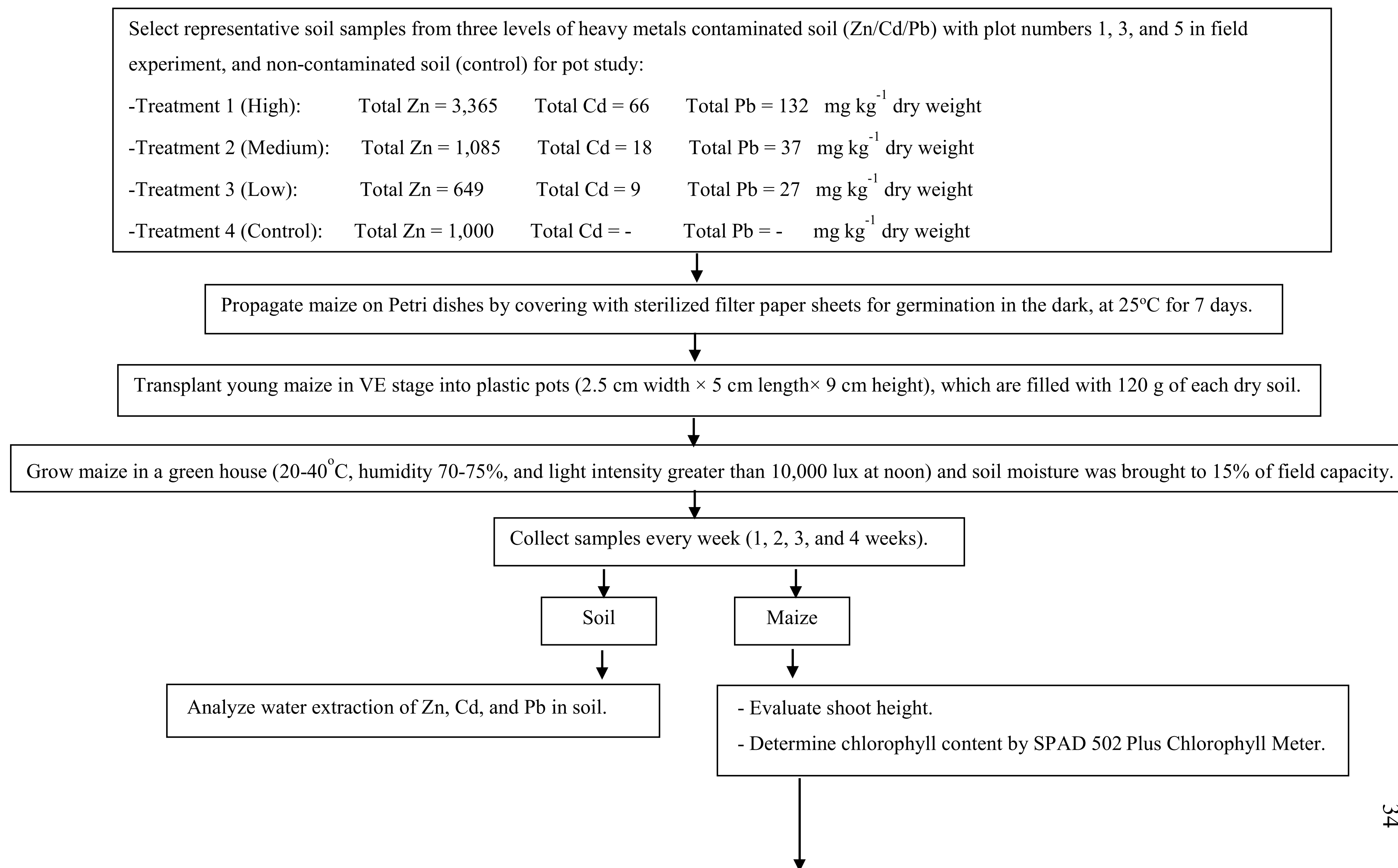
3.1.2 Pot experiments



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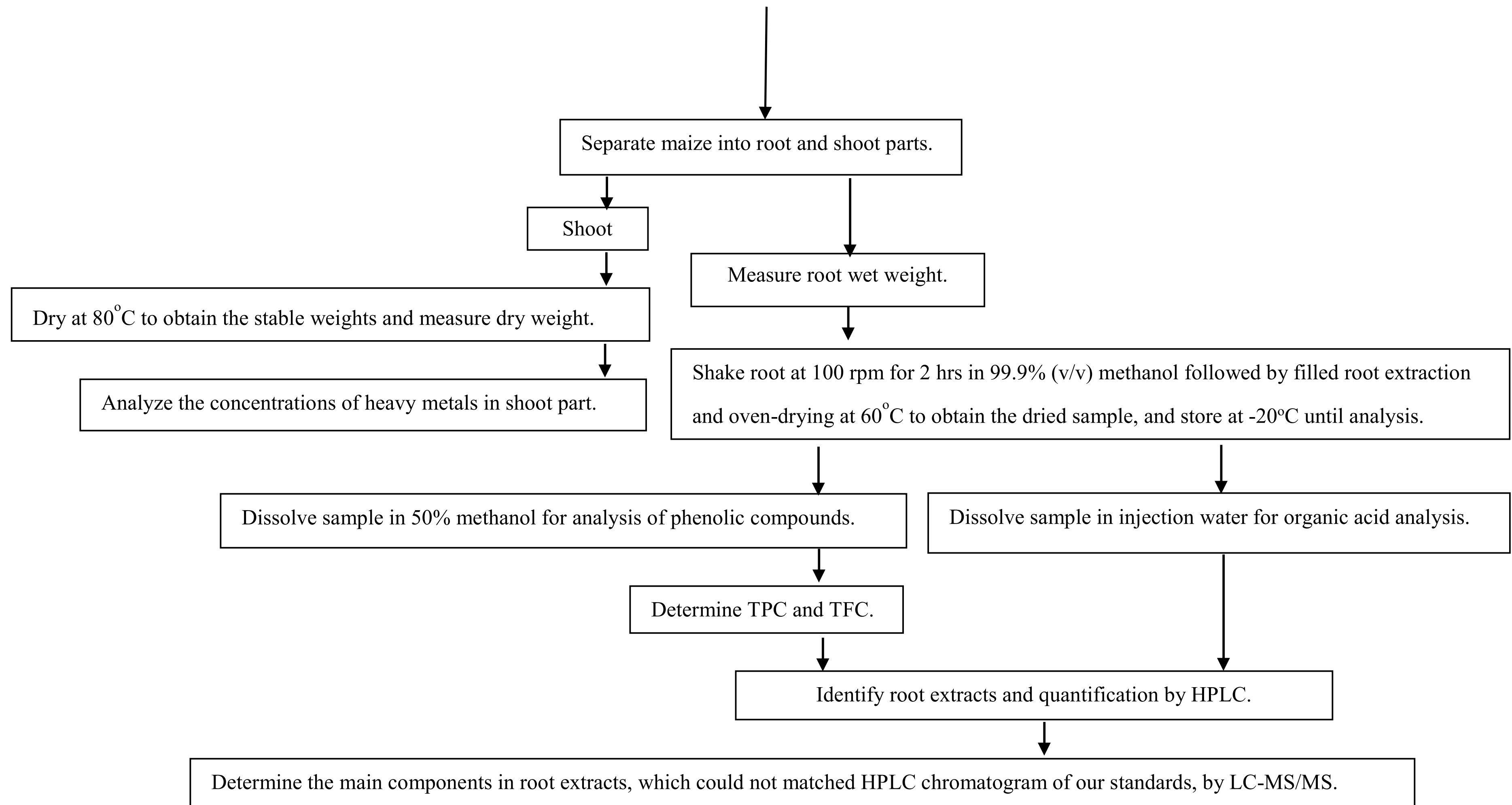

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3.2 Methods

3.2.1 Field experiment and soil sampling

The field site was located in an agricultural area near Mae-Tao Creek in Pha Te Village, Phatat Phadaeng Sub-district, Mae Sot District, Tak Province, Thailand (Appendix A-1). This field has been used to grow maize for many years. Irrigation and water run-off are primary sources of the observed elevated levels of heavy metals (Simmon *et al.*, 2005; Sebastian *et al.*, 2016). The metal concentrations and soil properties of the five plots before maize growing are shown in Table 3.1. The field experiment was carried out in a 1.5 m x 50 m area divided into five plots of 1.5 m x 10 m with each plot separated by a distance of 10 m (Appendix A-1). The soil texture was loamy sand.

The field experiment was conducted for four months during early and late rainy seasons (between June and October, 2013). Conservation practices for maize in Mae Sot included conservation tillage and harrowing for seedbed preparation. The amount of rainfall and average temperature in each month were acquired from the Thai Meteorological Department (Appendix A-2). Maximum and minimum rainfall were 640.5 mm month^{-1} and 31.8 mm month^{-1}, respectively. The maximum, minimum, and average temperatures were 33.2, 30.9, and 31.9 ºC, respectively. Hybrid maize seeds of TF 222, which is a drought tolerance maize in industrial agriculture (Changsaluk *et al.*, 2012), were purchased from Thai Seed Trade Association (THASTA), Thailand. The seeds were coated with fungicide. The maize seeds were sowed as 50 seeds per plot. The first, complete fertilizer (15-15-15) application was done simultaneously with sowing. The second, urea (46-0-0), was applied at 60 days after sowing without soil cover. Four plant samples were taken from each plot using a Randomized Complete Block Design (RCBD) following the Simple Random Sampling (SRS) technique by sampling without replacement method at the four stages of V10-tenth-leaf, R1-silking, R4-dough, and R6-maturity (Ciampitti *et al.*, 2016) (Appendix A-2). A plant with roots was carefully dug out as a ball with a 20 cm diameter and 20 cm deep. The root-soil systems were separately shaken vigorously in a sterile plastic container to collect bulk soil or root non-adhering soil. Then, rhizospheric soil was directly scraped from the root adhering soil with a disposal sterilized plastic spatula. However, the bulk and rhizospheric soils of maize in the VE stage could not be collected due to smaller amounts. The bulk and rhizospheric

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soils were immediately transferred to polyethylene bags and stored 4ºC during transportation. A subsample for DNA extraction was frozen at -20ºC.

Table 3.1 Characteristics of soils from five studied sites

Site	Position	Total concentration in soil (mg kg^{-1})		
		Zn	Cd	Pb
1	N 16º 40’ 26” E 98º 37’ 46”	4,883 ± 237	85 ± 2	154± 8
2	N 16º 40’ 25” E 98º 37’ 46”	2,693 ± 152	37± 3	76± 5
3	N 16º 40’ 25” E 98º 37’ 46”	1,403 ± 104	18± 1	45± 4
4	N 16º 40’ 25” E 98º 37’ 45”	1,287 ± 134	13± 0	38 ± 3
5	N 16º 40’ 24” E 98º 37’ 45”	379± 44	6± 1	34± 4

3.2.2 Soil texture and chemical analysis

A soil sample was dried at 80ºC for 24-48 hrs until a stable weight was reached to determine the soil moisture content. The dried soil was ground into a fine powder and sieved through a 2-mm nylon mesh sieve. Soil texture was determined by a simplified method for soil particle-size determination (Kettler *et al.*, 2001). A 1:1 soil:water suspension was shaken at 150 rpm for 1 h, and the values of pH and electrical conductivity (EC) were recorded by a pH meter (Denver Instrument Model 215 , USA) and an EC meter (Hanna HI 99301, Romania), respectively (Estefan *et al.*, 2013). Cation exchange capacity (CEC) was studied by leaching with ammonium acetate ($C_2H_7NO_2$) at pH 7 (Estefan *et al.*, 2013). Organic matter (OM) was measured by the loss of weight via the ignition method at 360ºC (Schulte and Hoskins, 1996). Potassium (K), phosphorus (P), and nitrogen (N) were analysed following the methods of the Land Development Department (LDD), Ministry of Agriculture and Cooperatives, Thailand. Analysis methods used by the LDD were Bray-II for extractable P (Bray and Kurtz, 1945), ammonium acetate extraction for extractable K (ICARDA, 2001), and micro Kjeldahl for total N (Black, 1965). All chemicals applied were analytical grade.

To determine total concentrations of Zn, Cd, Pb, and Fe in a dried soil sample, 0.1 g of each sample was digested in 3 ml of aqua regia (a 3:1 volume ratio of 37% (w/w) HCl and 69% (w/w) HNO_3) in an open tube digestion method (McGrath and Cunliffe, 1985). In case of extractable concentrations of the metals, the soil was shaken in 0.005 M

diethylene triamine penta acetic acid (DTPA) with a soil:extractant ratio of 1:2 at 120 rpm for 2 hrs (Lindsay and Norvell, 1978). The total and extractable metal concentrations were measured by a flame atomic absorption spectrophotometer (AAS) (Shimadzu AA-680, Japan) and an inductively coupled plasma optical emission spectrometer (ICP-OES) (PerkinElmer Optima 8000, USA).

3.2.3 Determination of bacterial counts and culturable bacterial community

A 5 g wet weight of each bulk and rhizospheric soil sample were suspended in 50 ml of sterile 0.85% (w/v) sodium chloride (NaCl) by shaking at 200 rpm for 2 hrs. Serial dilutions (10^{-1}-10^{-4}) were carried out with sterile deionized water. The samples diluted at 10^{-3} and 10^{-4} were studied in triplicate with the spread plate technique on nutrient agar (NA) (Himedia, India). The visible colonies of bacteria were enumerated and marked daily throughout the incubation periods of three days. Colony morphology was characterized based on colour, form, elevation, margin, surface, and optical properties (Harley and Prescott, 2002). The colonies were grouped into morphotypes and counted into colony forming unit (CFU). The cultivable heterotrophic and genetic diversity were investigated following Hu *et al.*, (2007) (Appendix A-6). The colony morphotypes that were commonly and rarely cultured from the rhizosphere of every maize growth stage, were picked up and isolated by a quadrant steak method (Harley and Prescott, 2002).

3.2.4 Isolation of genomic DNA from bacterial culture and PCR amplification

The bacterial isolates were identified by partial sequencing of the 16S rDNA genes. Each isolate was grown in 50 ml of nutrient broth (NB) and shaken at 30±2ºC for 24 hrs. The cells were collected by centrifugation and thrice washing with sterile 0.85% (w/v) NaCl. The bacterial pellet was resuspened in sodium chloride-Tris-EDTA (STE) buffer, added 1% (v/v) sodium dodecyl sulfate (SDS), then incubated at 90ºC for 5 min. Subsequently, the suspension was extracted with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) (modified method of Sambrook *et al.*, 1989). The aqueous phase was precipitated with 3 M sodium acetate (pH 5.2) and 99.8 % (v/v) ethanol at -20ºC for 30 min. The pellet was washed by 70 % (v/v) ethanol and air-dried before being resuspended in 50 µl 1x Tris-EDTA (TE) buffer. The DNA encoding the 16S rRNA was amplified with universal primers fD1 (5' AGAGTTTGATCCTGGCTCAG 3') and rP2 (5' ACGGCTACCTTGTTACGACTT 3')

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(Weisburg *et al.*, 1991), which yielded products of approximately 1,500 base pairs. The PCR reaction containing 15-50 ng of DNA template, 0.5 µM of each primer, 0.2 mM dNTP mix (Vivantis, Malaysia), 1x PCR buffer, 1.5 mM $MgCl_2$, and 2.5 units Taq DNA polymerase (Invitrogen, Brazil) was performed with a thermal cycler (Applied Biosystems Model 9902, USA). The thermal cycling program amplifications are shown in Table 3.2. The amplified PCR products were cleaned with a GF-1 PCR clean-up kit (Vivantis, Malaysia), and sequenced by Macrogen Inc, Korea. The sequence data of the 16S rDNA was compared with sequences in the National Center for Biotechnology Information (NCBI) database using the BLASTN 2.6.1 to locate nearly exact matches in the 16S ribosomal RNA sequences (bacteria and archaea) database (Zhang *et al.*, 2000). The most closely related sequences were aligned following the function of ClustalW multiple alignment by BioEdit version 7.1.9 (Hall, 1999). Phylogenetic trees were constructed by MEGA version 6 using the Neighbor-joining statistical method and Kimura-2-parameter model with 1000 bootstraps (Tamura *et al.*, 2013).

Table 3.2 PCR amplification condition for bacterial culture (Wood *et al.*, 1998)

Step	Temperature (ºC)	Time	Cycles
1	94	5 minutes	1
2	57	2 minutes	1
3	72	2 minutes	1
4	94	2 minutes	29
5	57	30 seconds	29
6	72	2 minutes	29
7	72	10 minutes	1

3.2.5 Total community DNA isolation and PCR-DGGE condition

DNA extraction from the soil samples was performed by a Power Soil DNA Isolation Kit (Mobio Laboratory, USA). A 0.8 g sample of each bulk soil or rhizospheric soil was processed according to the manufacturer's instructions. Genomic DNA yields were estimated from the DNA concentration by NanoDrop ND2000 (Thermo Scientific, USA), and visualized with 0.8% (w/v) agarose gel electrophoresis with ethidium bromide

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(EtBr) staining. The partial bacterial 16S rRNA genes were amplified with the forward primer 338F-GC (5'-CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGG GGAC TCCTACGGGAGGCA-3') and the reverse primer 518R (5'ATTACCGCGG CTGCTGG-3'), which yielded products of approximately 200 base pairs (Muyzer *et al*., 1993). The PCR reaction contained 20 ng of DNA template, 10 mM of each primer, 0.2 mM dNTPs, 3 mM $MgCl_2$, and 1 unit Taq DNA polymerase (Qiagen, Germany). The thermal cycling program amplification is shown in Table 3.3. PCR products were separated with 1.5% (w/v) agarose gel electrophoresis and EtBr staining. PCR products were separately subjected to DGGE analyzes by DGGE-2000 system apparatus (CBS Scientific Company, Del Mar, USA). Briefly, samples containing equal amounts of PCR products were loaded onto 8% (w/v) polyacrylamide gels in 1X TAE buffer with a denaturing gradient ranging from 40 to 65% denaturants (100% denaturant contains 7 M urea and 40% (v/v) formamide in 1X TAE). Electrophoresis was performed at 60 °C for 16 hrs at a constant voltage of 80 volts. The gel was stained with SYBR Gold nucleic acid stain (Invitrogen, USA) for 30 min, and the images were visualized on a UV transilluminator and captured using Biovision CN 1000/26M (Vilber Lourmat, France). Digital images of the gels were analyzed by Quantity One software (Biorad, USA). The presence (1) or absence (0) were scored as a binary matrix and clustered by unweighted pair grouping with the mathematical averages (UPGMA) method of the NTSYS-PC package for constructing a dendrogram (Rohlf, 2009).

The target DGGE bands were excised, resuspended in 20 µl of MilliQ water, and stored at 4ºC overnight. The DNA fragments recovered from the gel were used as templates for reamplification using primer 338F-GC-T7 (5'- *TAATACGACTCACTATA* CGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCA-3') and 518R using the primer binding sequences (T7 primer binding sites are in italics; GC clamp sequences are underlined) (O'Sullivan *et al*., 2008). The amplified PCR products were purified and sequenced by First BASE Laboratories Sdn Bhd, Malaysia. Sequences were generated and the most closely related sequences were obtained from the NCBI database.

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Table 3.3 PCR amplification condition for total bacterial community

Step	Temperature (ºC)	Time	Cycles
1	95	5 minutes	1
2	95	30 seconds	30
3	60	10 seconds	30
4	72	30 seconds	30
5	72	7 minutes	1

3.2.6 Plant analysis

Plants in the VE stage were not used for data analysis because their weights were too little. The whole plant samples in the four stages of V10, R1, R4, and R6 were separated into root and shoot parts. The root was carefully washed with an excess of tap water and the water was wiped off. The shoot was separated into stalk, leaves, male flower, baby corn, seed, and corncob (without husks and silk), depending on the maize growth stages. Before sampling and grinding, all divided parts were sun-dried and oven-dried at 80ºC to obtain stable weights. The ground samples were digested following the modified method of Miller (1998). Sequentially, 0.1 g of plant sample was soaked in 3 ml of 70% (v/v) HNO_3 for 24 hrs and heated at 150˚C for 1 h, then 1 ml of 70% (v/v) $HClO_4$ was added and heated at 215 ºC for 2 hrs, before adding 3 ml of deionized water and heating at 90ºC for 1 hr. The digested plant solutions were analyzed by AAS and ICP-OES.

3.2.7 Pot design and soil sampling

Maize seeds were propagated on Petri dishes. The seeds were covered with sterilized filter paper sheets, and 10 ml of sterilized distilled water was added to each Petri dish to moisten the filter paper sheets and allow the germination in the dark, at 25 ºC for seven days. After seeding growth, young maize at the VE stage were transplanted individually into plastic pots (2.5 cm width × 5 cm length× 9 cm height) (Appendix B-1) by separation into two groups for growth in the heavy metals contaminated soil sample and non-contaminated soil (control, CT) (Table 3.4). Three levels of heavy metals contaminated soil (Zn/Cd/Pb) were collected from the row spacing of maize grown in plot numbers 1, 3, and 5 in the field experiment because it showed the different Zn/Cd/Pb

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concentrations in the soil and exhibited different metals effecting maize growth. The non-contaminated soil (CT) without Cd and Pb was obtained from Ban Chiang Hian, Maha Sarakham, Thailand. The soil properties were analyzed as described in 3.2.2. A pot was filled with 120 g of each dry soil. The soil moisture was brought to 15% of field capacity. The maize seedlings were transplanted at the same depth (approximate 1 cm below the surface). The pot experiment was laid down in a random complete block design (RCBD) with three replications. The plants were grown in a green house (20-40ºC, 70-75% humidity and light intensity greater than 10,000 lux at noon). The samples were harvested in 7, 14, 21, and 30 days after planting. They were evaluated for height, dry weight, soil moisture, soil pH, and Zn and Cd accumulation in the shoot. Total chlorophyll content was determined by a SPAD 502 Plus Chlorophyll Meter (Spectrum Technologies, USA). Readings were taken from the center of the second leaf and whorl leaf (Figure 3.1).

Table 3.4 Soil properties, quantities of elements, and concentration of Zn, Cd, and Pb

Properties	Control (CT)	Treatment-1 (High)	Treatment-2 (Medium)	Treatment-3 (Low)
Total-Zn*	26.0±3.6	3,364.5±581.1	1,085.1±62.8	649.2±13.4
Total-Cd*	ND	65.5±5.1	18.0±2.6	9.1±1.7
Total-Pb*	ND	131.8±6.0	37.2±2.4	26.9±5.8
DTPA-Zn*	5.9±0.8	179.0±30.8	165.9±10.0	124.4±15.3
DTPA-Cd*	ND	9.7±0.4	6.4±1.2	5.4±1.7
DTPA-Pb*	ND	33.2±0.2	19.2±5.5	15.1±3.2
Quantities of elements (%)				
- Nitrogen (N)	0.84±0.28		0.60±0.36	
- Carbon (C)	4.55±1.47		2.94±0.43	
- Hydrogen (H)	0.16±0.02		0.27±0.05	
- Sulfur (S)	1.61±0.02		1.64±0.03	
- Oxygen (O)	8.03±0.07		5.54±0.73	
Soil texture		Loamy sand		

* Unit of metal concentration was mg kg^{-1} of soil dry wt.

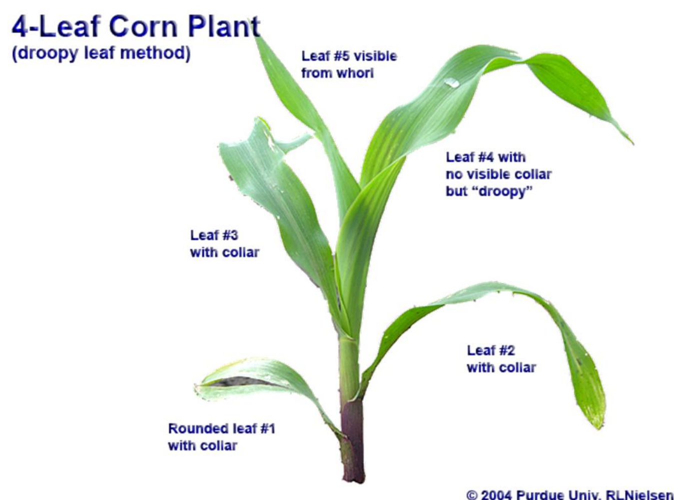

Figure 3.1 Determining maize leaf stages (Nielsen, 2004)

3.2.8 Water extraction of heavy metals in soil

To determine the water solubility of Zn, Cd, and Pb in an 80ºC dried soil sample, 0.5 g of each sample was shaken in a 15 ml centrifuge tube with 10 ml of deionized water for 2 hrs (Cajuste *et al.,* 2000). The solutions from each sample were centrifuged at 8,000 rpm for 10 min and filtered through Whatman no. 5 filter paper. The water soluble levels of the heavy metals were measured by a flame atomic absorption spectrophotometer (AAS) (Shimadzu AA-680, Japan).

3.2.9 Root extraction

The plant samples of 7, 14, 21, and 30 days were separated into root and shoot parts. The root was carefully washed with an excess of tap water and the water was wiped off. The exudate from the apoplast and cell wall freespace of the root were extracted by 99.9% (v/v) methanol (Chaffai *et al.,* 2006; Hao *et al.,* 2007). Root samples were shaken at 100 rpm for 2 hrs in 99.9% (v/v) methanol and filtered through Whatman no. 5 filter paper into a sterile tube. The methanolic extracts containing the organic acids and phenolic compounds were evaporated by oven-drying at 60ºC to obtain the dried sample and stored at -20ºC until analysis. The samples were assayed for total phenolic content (TPC) and total flavonoid content (TFC). Phenolic compounds and organic acids were

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determined by high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectroscopy/mass spectroscopy (LC-MS/MS).

3.2.10 Total phenolic content (TPC) and total flavonoid content (TFC)

Total phenolic content was determined by a modified Folin-Ciocalteau method (Cicco *et al.,* 2009). TPC analysis was performed using Folin-Ciocalteu reagent (Carlo Erba Reactifs SA, USA), sodium carbonate (Na_2CO_3) (Ajax Finechem, Australia), and 99.9% (v/v) methanol. Gallic acid ($C_7H_6O_5 \cdot H_2O$) (Sigma-Aldrich, China) was chosen as a standard phenol. Root extract samples were dissolved in 1 ml of 50% (v/v) methanol. 100 µl of the sample was pipetted into 1.5 ml microcentrifuge tubes and 500 µl of 10% (v/v) Folin-Ciocalteau reagent was applied. The mixture was left to stand in the dark for 3 min and then 400 µl of 7.5% (w/v) Na_2CO_3 was added. After 30 min in the dark, the absorbance was determined at 731 nm using a UV/visible spectrometer (Beckman Coulter DU 730 Life Science, USA). A standard curve was prepared from 10, 20, 40, 60, 80, and 100 mg l^{-1} of gallic acid. TPC was expressed in terms of a gallic acid equivalent (mg GAE g^{-1} wet wt.).

Total flavonoid content was analyzed using a colorimetric method (Pekal and Pyrzynska, 2014). Briefly, 500 µl of deionized water and 100 µl of the sample were added to 1.0 ml microcentrifuge tubes cover by aluminum foil. Then, 30 µl of 5% (w/v) $NaNO_2$ was added. The mixtures were left in the dark for 5 min before 60 µl of 10% (w/v) $AlCl_3$ was added. After standing for 6 min, 200 µl of 1 M NaOH and 110 µl of deionized water were added. After 5 min in the dark, the absorbance was measured at 510 nm. A standard curve was prepared from 10, 20, 25, 50, 100, 250, and 500 mg l^{-1} of epicatechin. TFC was expressed in terms of an epicatechin equivalent (mg EPE g^{-1} wet wt.).

3.2.11 Detection and quantification of root extracts by HPLC

The root extracts of phenolic compounds, flavonoid compounds, and organic acids were determined using HPLC (Shimadzu SIL-10AD, Japan) with a C18 guard column (4.6 mm x 10 mm, 5 µm) (VetiSep^{TM} UPS C-18, Thailand) and a C-18 reversed-phase column (4.6 mm x 250 mm, 5 µm) (GL Science Lab InertSustain C-18, Japan).

For phenolic compounds and flavonoid compounds, root extract samples were dissolved in 1 ml of 50% (v/v) methanol and filtered through a 0.45 mm nylon filter (Whatman, GE Healthcare, UK), and then they were injected into column C-18 with an injection volume of 20 µl. The gradient mobile phase was performed by varying the

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proportion of solvent A (deionized water-acetic acid, 97:3 v/v) to solvent B (99.9% (v/v) methanol), with a flow rate of 1 ml min^{-1} (Zuo *et al*., 2002). 100% (v/v) glacial acetic acid (Merck, Germany) and 99.9% (v/v) methanol HPLC grade (VMR international, UK) were applied. The gradient percentage ratios of solvent A:solvent B were as follows: 100:0, 0-5 min; 90:10, 5-10 min; 80:20, 10-15 min; 70:30, 15-20 min; 60:40, 20-30 min; 50:50, 30-40 min; 40:60, 40-50 min; and post-run 100:0, 50-55 min before next injection. The photodiode array (PDA) detector acquisition wavelength was set in the range of 200-400 nm and the outputs at 254, 280, and 360 nm were analyzed (Mongkhonsin *et al.,* 2016). Reference chemical standards of phenolic compounds and flavonoids were gallic acid ($C_7H_6O_5{\cdot}H_2O$) (Sigma-Aldrich, China), quercetin dihydrate ($C_{15}H_{10}O_7{\cdot}2H_2O$) (Fluka, Germany), myricetin ($C_{15}H_{10}O_8$) (Fluka, France), catechin ($C_{15}H_{14}O_6$) (Fluka, Switzerland), kaempferol ($C_{15}H_{10}O_6$) (Fluka, Germany), epicatechin ($C_{15}H_{14}O_6$) (Fluka, France and Germany), caffeic acid ($C_9H_8O_4$) (Sigma-Aldrich, Switzerland), vanillin (C_8H_8O3) (Carlo Erba, France), naringenin ($C_{15}H_{12}O_5$) (Sigma-Aldrich, UK), chlorogenic ($C_{16}H_{18}O_9$), wedelolactone ($C_{16}H_{10}O_7$) (Calbiochem, Germany), p-coumalic ($C_6H_4O_4$), and rutin ($C_{27}H_{30}O_{16}$) (Merck, UK). The identification of each compound was based on a combination of retention times.

For organic acid, root extract samples were dissolved in 1 ml of injection water, filtered through a 0.45 mm nylon filter, and injected into column C-18 with an injection volume of 20 µl. The mobile phase was performed by varying the proportion of solvent A 0.1% of H_3PO_4 (v/v) (deionized water-phosphoric acid) to solvent B (99.9% (v/v) methanol) at a flow-rate of 1 ml min^{-1} and maintained at a temperature of 30 °C (condition from GL science, Data No. LB109-0919). The percentage ratios of solvent A:solvent B were 98/2 (v/v). The photodiode array (PDA) detector acquisition wavelength was set in the range of 200-400 nm and the outputs at 214 nm were analyzed. The identification of each compound is based on a combination of retention time between sample solution and standard solution with reference standards of citric acid ($C_6H_{10}O_8$) (Sigma-Aldrich, Germany), maleic acid ($C_4H_4O_4$) (Fluka, USA), malic acid ($C_4H_6O_5$) (Fluka, USA), oxalic acid ($C_2H_2O_4$) (Ajax Finechem, Australia), and succinic acid ($C_4H_6O_4$) (Ajax Finechem, Australia).

3.2.12 Identification of root extracts by LC-MS/MS

The main components in the root extracts that could not be matched with a standard chemical in the HPLC chromatogram were determined by LC-MS/MS, with quadrupole-time of flight (Q-TOF) mass analyzers. The LC-QTOF-MS/MS analysis was performed on an Agilent HPLC 1260 series coupled with a QTOF 6540 UHD accurate mass (Agilent Technologies, Waldbronn, Germany). The separation of the sample solution was performed on a Luna C18(2) 150x4.6 mm, 5 µm (Phenomenex, USA). Phenolic compounds in the root extracts were identified by LC-QTOF-MS/Ms following the method of Mongkhonsin *et al.,* (2016). The solvent flow rate was 500 µl min^{-1} and 5 µl of the sample solution was injected into the LC system. The binary gradient elution system was composed of water as solvent A and acetonitrile as solvent B, and both contained 0.1% formic acid (v/v). The linear gradient elution was 5-95% for solvent B at 35 min and a post run for 5 min. The column temperature was set at 35 ºC. The conditions for the negative ESI source were as follows: drying gas (N_2) flow rate 10 L min^{-1}, drying gas temperature 350 ºC, nebulizer 30 psig, fragmentor 100 V, capillary voltage 3500 V, and scan spectra from m/z 100-1500 amu. The auto MS/MS for the fragmentation was set with collision energies of 10, 20, and 40 V. All data analyses were controlled using Agilent MassHunter Qualitative Analysis Software B06.0 (Agilent Technologies, CA, USA).

3.2.13 Statistical analysis

The data were reported as the means ± standard deviations (SD) and analysed using paired sample *t*-test (*T test, $P<0.01$*), box plot, and analysis of variance (ANOVA). Significant differences between the means were determined by Duncan’s new multiple range test (DMRT) at $P< 0.01$. Spearman’s rank correlation coefficients were determined to compare the correlations between the various parameters ($P<0.05$ and $P< 0.01$). Statistical analyses were performed using SPSS statistical software (SPSS 14, SPSS Inc., IL, USA). The principle component analysis (PCA) plots were generated by a correlation matrix using the PAST v3.14 software.

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CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Characteristics of soil with planting stage

The characteristics of the soil and soil bacterial community collected from the field at the VE stage are presented in Table 4.1. The cultivable heterotrophic and genetic diversity, Shannon-Wiener indices (*H*), richness (*S*), and evenness (E_H) were investigated following equations (1) and (2), respectively:

$$H = - \sum_{i=1}^{S} Pi \ln Pi = - \sum_{i=1}^{S} \left(\frac{Ni}{N}\right) \ln\left(\frac{Ni}{N}\right) \quad (1)$$

$$E_H = H/H_{max} = H/\ln S \quad (2)$$

Where P_i is the ratio between the number in a specific group (N_i) and the total number (*N*) while *S* is the total number of morphotypes in the cultivable heterotrophic diversity.

Table 4.1 shows that the percentage soil moisture, extractable Zn (DTPA-Zn), and bacterial community in the soil were not significantly different across the area studied ($P>0.01$), but the other parameters were significantly different ($P<0.01$). The levels of soil classification were determined following the Land Development Department (LDD), Ministry of Agriculture and Cooperatives, Thailand. The pH values were 6.33 to 7.49, but the majority of samples were light acidic soil. The electrical conductivity (EC) values were classified as low level (EC<300 µs cm^{-1}). The organic matter (OM), available P, available K, and total N in samples were classified as high content (mean values OM>3.5%, P>20 mg kg^{-1}, K>90 mg kg^{-1}, N>0.17%). The cation exchange capacity (CEC) showed a medium content (mean value 10<CEC<20 cmol kg^{-1}). The chemical soil properties in the VE stage showed soil fertility. Under the conservation practices for maize in Mae Sot, including conservation tillage, harrowing for seedbed preparation, and applying complete fertilizer (15-15-15) with sowing, which caused high nutrients in the soil. Generally, maize can be grown successfully on soils with a pH of 5.8-7.0, but a moderate acid environment of pH 6.0-7.0 is optimum (Land Development Department,

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1998; The Potash Development Association, 2008). However, the content of metals in soil followed the ranking 302-3,132 mg Zn kg^{-1}, 5-66 mg Cd kg^{-1}, 30-136 mg Pb kg^{-1}, and 9,070-18,108 mg Fe kg^{-1}. The majority of Cd, Pb, and Zn concentrations in soil were higher than the maximum allowable concentrations (MAC) in agricultural soils with 1-5 mg Cd kg^{-1}, 100-300 mg Pb kg^{-1}, and 50-100 mg Zn kg^{-1}, respectively (Kabata-Pendias, 2011). Due to this area being a source of Zn mineralization, Cd and Pb are mostly “guest” metals positively correlated with Zn mineralization (Purves, 1985). Soil samples from agricultural areas around the Pha Te Village, Mae Sot District, have total soil Cd and Zn concentrations ranging from 0.63-30.4 mg Cd kg^{-1} and 14.4-594 mg Zn kg^{-1}, respectively (Akkajit, 2015). The extractable concentration of Zn, Cd, Pb, and Fe in soils were 63-101 mg Zn kg^{-1}, 4-13 mg Cd kg^{-1}, 11-29 mg Pb kg^{-1}, and 38-104 mg Fe kg^{-1}, respectively.

The culturable bacterial community was shown in terms of richness, eveness, and Shannon-Wiener index. The results showed that the culturable bacterial communities in the VE stage were not significantly different when compared to the different levels of the heavy metals contaminated agricultural soils ($P>0.01$). This result indicated that in this initial study there were the same bacterial communities in each plot. The richness, eveness, and Shannon-Wiener index were ranked as 11-13, 0.74-0.87, and 1.86-2.71, respectively. The mean of the Shannon-Wiener index was lower than the typical values of the Shannon-Wiener index (1.5 to 3.5) in most ecological studies (Magurran, 2004). Nihorimbere *et al.,* (2011) reported that only 2% of soil microbes can be cultured. Heavy metals strongly reduced the numbers and species diversity of the soil microbial communities, but enhanced the development of metal-resistant microbial populations (Yao *et al.,* 2003). The high concentrations of heavy metals (Zn, Cd, and Pb) in this area are toxic to soil microbes (Kabata-Pendias, 2011). Total bioactivity, richness, and diversity of microorganisms decreased with increasing heavy metal concentrations because the microorganisms differed in their sensitivity to heavy metal toxicity (Xie *et al.,* 2016). The results of this study indicated that bacterial communities growing in the heavy metal contaminated sites could tolerate high concentrations of metals.

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Table 4.1 Chemical properties and bacterial diversity of soils collected from field site at VE stage of maize growth (n=3)

Parameters	Plot-1	Plot-2	Plot-3	Plot-4	Plot-5
pH	7.49±0.16^{a}	6.33±0.11^{b}	6.49±0.08^{b}	6.52±0.09^{b}	6.46±0.10^{b}
EC (µs cm^{-1})	191.67±18.93^{a}	165.50±12.38^{a}	95.30±4.29^{b}	79.17±8.84^{b}	72.50±7.31^{b}
Om (%)	6.39±0.91^{ab}	5.60±0.52^{bc}	4.39±0.49^{cd}	3.64±0.32^{d}	3.48±0.43^{d}
P (mg kg^{-1})	20.17±0.34^{a}	24.89±0.19^{b}	17.71±0.32^{c}	30.21±0.23^{d}	26.53±0.19^{e}
K (mg kg^{-1})	117.00±4.20^{a}	98.23±2.35^{b}	88.63±1.24^{c}	101.57±0.53^{b}	117.74±1.37^{a}
N (%)	0.28±0.05^{a}	0.23±0.01^{ab}	0.23±0.02^{ab}	0.21±0.03^{ab}	0.20±0.03^{b}
CEC (cmol kg^{-1})	9.00±0.15^{a}	12.57±0.17^{b}	10.10±0.11^{c}	9.75±0.43^{c}	8.56±0.02^{a}
Moisture (%)	1.93±0.10^{a}	1.95±0.12^{a}	1.73±0.23^{a}	1.77±0.10^{a}	1.80±0.13^{a}
Total-Zn (mg kg^{-1})	3,123.00±151.41^{a}	1,722.29±97.28^{b}	897.50±66.68^{c}	823.11±85.55^{c}	302.31±105.96^{d}
Total-Cd (mg kg^{-1})	66.06±2.94^{a}	27.85±2.86^{b}	10.50±0.23^{c}	8.19±0.90^{c}	5.55±0.98^{c}
Total-Pb (mg kg^{-1})	136.83±3.90^{a}	76.01±5.09^{b}	44.55±4.09^{c}	42.58±7.49^{c}	30.09±1.20^{d}
Total-Fe (mg kg^{-1})	18,108.40±72.20^{a}	15,984.96±775.40^{b}	11,527.04±1038.17^{c}	9,540.01±403.57^{d}	9,070.22±134.99^{d}
DTPA-Zn (mg kg^{-1})	101.01±20.77^{a}	65.46±2.86^{a}	62.68±21.07^{a}	68.11±20.19^{a}	98.45±5.70^{a}
DTPA-Cd (mg kg^{-1})	13.09±1.50^{a}	3.83±0.16^{b}	8.01±1.33^{d}	6.69±0.86^{cd}	4.65±0.57^{bc}
DTPA-Pb (mg kg^{-1})	28.70±2.44^{a}	28.77±1.68^{b}	18.80±0.55^{c}	15.12±1.10^{cd}	10.89±2.59^{d}
DTPA-Fe (mg kg^{-1})	56.73±0.68^{a}	38.03±1.78^{b}	64.48±0.87^{a}	104.18±1.81^{c}	54.99±10.70^{a}
Log CFU/g soil dry wt.	8.67±0.17^{a}	8.74±0.02^{a}	8.60±0.15^{a}	8.72±0.05^{a}	8.69±0.03^{a}
Richness	12±2^{a}	13±2^{a}	11±2^{a}	12±1^{a}	13±2^{a}
Evenness	0.87±0.05^{a}	0.76±0.04^{a}	0.82±0.07^{a}	0.75±0.06^{a}	0.74±0.11^{a}
Shannon-Wiener index	2.17±0.12^{a}	1.91±0.04^{a}	1.96±0.04^{a}	1.86±0.09^{a}	1.89±0.35^{a}

Different letters (a-e) in the same row show significant differences (*P<0.01*). The data are given as means±SD (n=3).

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4.2 Field experiment

4.2.1 Comparison between bulk soil and rhizospheric soil

After the VE stage, the results showed that both the bulk soil and rhizospheric soil had slightly different total concentrations of heavy metals. However, the extractable heavy metals tended to increase in the maize growth stages (V-10, R-1, R-4, and R-6) (Appendix E-F). A comparison of the properties of abiotic factors (i.e., pH, OM, metals concentration, etc.) and culturable bacterial community between bulk soil and rhizospheric soil are presented in Table 4.2 (Appendix G). Comparing the means of bulk soil and rhizospheric soil were showed in terms of paired sample tests. The results showed that EC, OM, total heavy metals content, extractable concentrations of Zn, and richness were not significantly different between bulk soil and rhizospheric soil with various distances and stages of maize development (*T-test,* $P>0.01$). On the other hand, pH, P, K, N, CEC, extractable concentrations of Cd, Pb and Fe, number of heterotrophic bacteria, evenness, and Shannon-Wiener index were significantly different between bulk soil and rhizospheric soil (*T-test,* $P<0.05$ and $P<0.01$). High pH, CEC, evenness, and Shannon-Wiener index were found in bulk soil. To produce a good maize required nutrition for normal plant growth and development, especially N, P, and K. The N take up rapidly from 40 days after sowing until about two weeks after flowering, whereas the K requirement increased at the end of flowering, and N+P continue until near maize maturity (NSW, 2009). Potassium (K) is the nutrient required in the greatest amount by maize (The Potash Development Association, 2008). It was involved in the energy production, maintaining osmotic potential, and nutritional status in plant, and taking up excess K in plants provided organic acid contents in the plant (Jones, 1998). Under flooded condition, soil microbes generated N_2O and transformed the form of the nitrogen in the soil, and there is high availability of N (Metz *et al.*, 2007). Plant physiology, growth of roots, root metabolism, and microorganisms could modify the bioavailable of micro- and macronutrients, pollution metals, and structure of microbial populations (Qureshi *et al.,* 2003; Wenzel *et al.,* 2004). Plant species and plant metabolic stress on the rhizobacteria, were able to select a specific rhizobacteria (Smalla *et al.,* 2001; Da Mota *et al.,* 2008). Johnston-Monje *et al.,* (2016) showed that

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Table 4.2 Comparison of bulk and rhizospheric soil in soil properties and Shannon-Wiener index by using paired samples *t*-test

No.	Paired sample	Parameters	Paired sample test				Paired sample correlations	
			N*	Paired differences mean	t	Sig.	Correlation	Sig.
1	Rhizosphere & Bulk	pH	60	-0.133	-2.962	0.002**	0.768	<0.001
2	Rhizosphere & Bulk	Ec	60	4.483	1.321	0.096	0.844	<0.001
3	Rhizosphere & Bulk	Om	60	0.081	1.126	0.132	0.742	<0.001
4	Rhizosphere & Bulk	P	60	3.323	5.387	<0.001**	0.768	<0.001
5	Rhizosphere & Bulk	K	60	33.588	8.085	<0.001**	0.602	<0.001
6	Rhizosphere & Bulk	N	60	0.009	4.226	<0.001**	0.592	<0.001
7	Rhizosphere & Bulk	CEC	60	-0.938	-3.447	0.001**	0.501	<0.001
8	Rhizosphere & Bulk	Total-Zn	60	0.321	0.866	0.145	0.771	<0.001
9	Rhizosphere & Bulk	Total-Cd	60	-57.165	-0.802	0.213	0.790	<0.001
10	Rhizosphere & Bulk	Total-Pb	60	-0.234	-0.521	0.302	0.979	<0.001
11	Rhizosphere & Bulk	Total-Fe	60	0.223	0.148	0.442	0.931	<0.001
12	Rhizosphere & Bulk	DTPA-Zn	60	-66.590	-0.207	0.418	0.734	<0.001
13	Rhizosphere & Bulk	DTPA-Cd	60	7.627	2.111	0.020*	0.431	0.001
14	Rhizosphere & Bulk	DTPA-Pb	60	1.743	3.371	0.001**	0.693	<0.001
15	Rhizosphere & Bulk	DTPA-Fe	60	1.530	1.991	0.026*	0.604	<0.001
16	Rhizosphere & Bulk	Moisture	60	16.800	1.791	0.039*	0.637	<0.001
17	Rhizosphere & Bulk	Log-CFU dry wt	60	0.267	5.907	<0.001**	0.391	0.002
18	Rhizosphere & Bulk	Richness	60	-0.383	-0.506	0.307	0.688	0.000
19	Rhizosphere & Bulk	Evenness	60	-0.199	-7.500	<0.001**	0.009	0.945
20	Rhizosphere & Bulk	Shannon-Wiener index	60	-0.525	-6.894	<0.001**	0.084	0.523

Superscript * shows number of samples comparing between bulk soil and rhizospheric soil

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bacterial diversity in rhizospheres of the commercial hybrid maize was less than bacterial diversity in the landrace maize. In addition, paired sample correlations determined the relationships between bulk soil and rhizospheric soil, in which the eveness and Shannon-Wiener index of bacterial communities had no correlations between the two groups of soils *(r=0.009* and *r=0.084; P>0.01*).

4.2.2 Correlation of bulk soil characteristics, temperature, rainfall, and culturable bacterial community

The result was obtained from a comparison of the characteristics of bulk and rhizospheric soil showed that Shannon-Wiener index were not correlated between bulk and rhizospheric soil. Therefore, the correlations between various distances from the irrigation source of Mae Tao Creek, stages of maize development, characteristics of soils, environmental parameters, and soil bacterial communities in both soil types were separately investigation.

A total of 60 bulk soil samples were collected from an agricultural area. Principal components analysis (PCA) was applied to analyze the relationships between abiotic factors and culturable bacterial community of bulk soil, as shown in Figure 4.1. This method reduced the dimensionality of a dataset, and it maintained the data variability information. Two principal factors explained 57.89% of the total inter-site variance parameters. The number of heterotrophic bacteria (CFU) and richness (Rich) showed positive relations to rainfall (Rain), but a negative relation to the stages of maize growth (Time) and temperature (Temp). The number species of soil bacteria were significantly correlated with cumulative temperature and rainfall in different agroclimatic zones (Manoharachary and Mukerji, 2006). Soil, water, temperature, and irrigation were considered for favourable microbial activity (Nogueira *et al.,* 2011). A single raindrop can transfer 0.01% of the bacteria on the soil surface, and bacteria transfer by rain is highly dependent on the regional soil profiles and climate conditions (Joung *et al.,* 2017).

The culturable bacterial community (Shannon) showed more related characteristics to soil, especialy metals contamination in agicultural soil. On the other hand, various distances from the irrigation source of Mae Tao Creek (Dis) showed a negative relation to metals contamination in agicultural soil and Shannon-Wiener index. Much research has shown that heavy metals had direct effects to soil microbial

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communities (Frostegard *et al.,* 1996; Giller *et al.,* 1998; Li *et al.,* 2006; Chien *et al.,* 2008). Hu *et al.,* (2007) showed heavily polluted soils could be characterized by the different structures of dominating bacteria by increasing the dominating bacterial community when Pb and Cd decreased. The majority of culturable bacterial communities in bulk soil correlated with the gradual concentrations of heavy metals due to the bacteria in bulk soil affected directly on the heavy metals, whereas microbes in the rhizosphere were protected by plants (Marschner *et al.,* 2004).

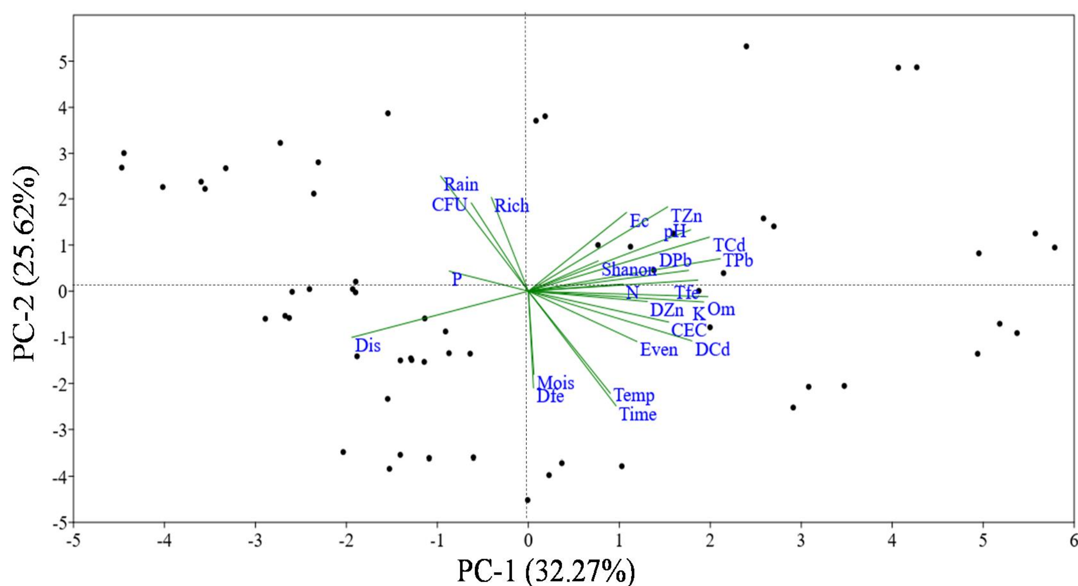

Figure 4.1 Principal component analysis of abiotic factors and bacterail community in bulk soil. (Dis, distances from irrigation source of Mae Tao Creek; Time, maize growth stages; Rain, rainfall; Temp, temperature; Ec, electrical conductivity; OM, organic matter; P, phosphorous; K, potassium; N, nitrogen; CEC, cation exchange capacity; Mois, percentage of soil moisture; TZn, TCd, TPb, and TFe, total concentrations of metals in soil; DZn, DCd, DPb, and DFe, extractable concentration of metals in soil; CFU, number of heterotrophic bacteria; Rhic, richness; Even, evenness; Shanon, Shannon-Weiner index) (n=60)

Based on the linear correlation, the Spearman's rank correlation coefficients showed the main factors in Table 4.3. The bioavailability of heavy metals in bulk soil was correlated to rainfall and growth stages. Watcharamai and Saenton (2013) reported that percentages of leaching of heavy metals in agriculture soil at Mae Tao were 0-1.38% of

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Zn, 0.06-0.29% of Cd, and 6.12-17.11% of Pb. The Cd and Zn contamination in this area was associated with the suspension of sediment by irrigation supply (Simmons *et al.,* 2005). Department of Primary Industries and Mines (2009) reported higher heavy metals contamination in agricultural soils around Mae Tao Creeks in the rainy season than in the summer season. Generally, bioavailable metals in soil tended to increase with a high soil moisture and equilibrium with cation exchange sites (Sheoran *et al.,* 2016). In addition, the water content in soil was a major contributor of geochemical cycling of elements (Brown *et al.,* 1999).

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Table 4.3 Spearman’s rank correlation coefficient between abiotic factors, rainfall, temperature, and culturable bacterial community with various distance from irrigation source and time of maize growth stages for bulk soil (n=60)

	Distance	Time	Rain	Temp	pH	Ec	Om	P	K	N	CEC	Mositure	TZn	TCd
Distance	1.000	0.000	0.000	0.000	-.789(**)	-.640(**)	-.597(**)	.298(*)	-.606(**)	-.331(**)	-.284(*)	0.203	-.608(**)	-.746(**)
Time		1.000	-1.000(**)	.949(**)	-0.109	-.435(**)	.326(*)	-.298(*)	.343(**)	0.122	.361(**)	.500(**)	-.388(**)	-0.028
Rain			1.000	-.949(**)	0.109	.435(**)	-.326(*)	.298(*)	-.343(**)	-0.122	-.361(**)	-.500(**)	.388(**)	0.028
Temp				1.000	-0.016	-.487(**)	.255(*)	-.331(**)	.340(**)	0.223	.382(**)	.314(*)	-.357(**)	-0.086
pH					1.000	.487(**)	.453(**)	-.283(*)	.440(**)	.359(**)	.476(**)	-.415(**)	.565(**)	.701(**)
Ec						1.000	.404(**)	0.217	.438(**)	0.074	0.059	-0.204	.626(**)	.668(**)
Om							1.000	-0.069	.790(**)	.360(**)	.370(**)	.301(*)	.417(**)	.730(**)
P								1.000	0.058	-0.098	-0.163	0.118	-0.111	-0.082
K									1.000	.495(**)	.280(*)	0.228	.334(**)	.654(**)
N										1.000	0.198	-0.031	0.232	0.232
CEC											1.000	-0.251	0.228	.469(**)
Mositure												1.000	-.373(**)	-0.108
TZn													1.000	.656(**)
TCd														1.000
TPb														
TFe														
DZn														
DCd														
DPb														
DFe														
CFU														
Even														
Rich														
Shannon														

Superscripts * and ** show significant differences at *P<0.05* and *P<0.01*, respectively

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Table 4.3 (cont')

	TPb	TFe	DZn	DCd	DPb	DFe	CFU	Even	Rich	Shannon
Distance	-.728(**)	-.684(**)	-0.108	-0.224	-.305(*)	0.203	-0.166	-.257(*)	0.030	-0.219
Time	0.136	0.155	.346(**)	.711(**)	0.226	.697(**)	-.578(**)	.392(**)	-.591(**)	-0.033
Rain	-0.136	-0.155	-.346(**)	-.711(**)	-0.226	-.697(**)	.578(**)	-.392(**)	.591(**)	0.033
Temp	0.043	0.133	.427(**)	.682(**)	.375(**)	.554(**)	-.402(**)	.310(*)	-.423(**)	0.005
pH	.620(**)	.632(**)	0.189	0.165	.363(**)	-.343(**)	.376(**)	0.085	0.121	0.178
Ec	.616(**)	.453(**)	0.001	-0.065	0.115	-.291(*)	.273(*)	0.076	0.194	0.168
Om	.815(**)	.712(**)	.291(*)	.456(**)	.291(*)	0.219	-0.172	.307(*)	-0.232	0.176
P	-0.123	-0.249	-.259(*)	-0.223	-0.143	-0.236	0.003	-0.242	0.170	-0.161
K	.714(**)	.627(**)	.261(*)	.378(**)	.391(**)	0.161	-0.113	.276(*)	-0.121	0.180
N	0.247	.336(**)	0.225	0.082	.299(*)	-0.055	0.159	-0.012	-0.057	0.030
CEC	.477(**)	.469(**)	.532(**)	.624(**)	.500(**)	-0.082	0.133	0.180	-0.171	0.139
Mositure	0.072	-0.027	-0.135	0.185	-.307(*)	.712(**)	-.751(**)	0.227	-.396(**)	-0.058
TZn	.605(**)	.542(**)	0.178	0.056	.325(*)	-.491(**)	.514(**)	-0.080	.333(**)	.260(*)
TCd	.951(**)	.772(**)	.300(*)	.336(**)	.353(**)	-0.160	0.167	0.195	-0.068	0.173
TPb	1.000	.773(**)	.355(**)	.471(**)	.357(**)	0.000	-0.021	.281(*)	-0.202	0.138
TFe		1.000	.279(*)	.332(**)	.379(**)	-0.004	0.098	.266(*)	-0.180	0.112
DZn			1.000	.727(**)	.843(**)	0.012	-0.011	.279(*)	-0.136	0.139
DCd				1.000	.640(**)	0.225	-.297(*)	.379(**)	-.380(**)	0.047
DPb					1.000	-0.250	0.191	0.130	0.071	0.108
DFe						1.000	-.659(**)	.296(*)	-.558(**)	-0.102
CFU							1.000	-.591(**)	.600(**)	-0.087
Even								1.000	-.391(**)	.589(**)
Rich									1.000	.333(**)
Shannon										1.000

Superscripts * and ** show significant differences at *P<0.05* and *P<0.01*, respectively

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4.2.3 Correlation of abiotic factors, biotic factors, and culturable bacterial community in rhizospheric soil

The PCA of the abiotic factors, various distances from the irrigation source of Mae Tao Creek, metals accumulation in plants, maize growth stages, and culturable rhizobacterial communities in rhizospheric soil (n=60) are shown in Figure 4.2. Two principal factors interpletation showed 54.20% of the total inter-site variance parameters. The distances were negatively correlated with the total and extracable concentrations of the metals (Zn, Cd, Pb, and Fe), but they were not correlated with the culturable rhizobacterial communities, total dry weight of maize (Total), and metals accumulation in maize (Root-Zn, Root-Cd, and Shoot-Zn). The culturable rhizobacterial communities in the rhizosphere had positive correlations with the metals accumulation and rainfall, but they had a negative correlation with maize growth stages, temperature, soil moiture, and total plant dry weight. The results indicated that the rhizobacterial communities were an important factor for metals accumulation in maize. The heavy metals contamination in the rhizospheric soil did not affect the rhizobacterial communities, whereas the maize growth stages were the main effect on the communities.

The microbes in the soil are diverse and different in quantity and quality due to climatic, edaphic, and biotic factors (Manoharachary and Mukerji, 2006). Progression of plant growth stages related to environmental changes, such as soil temperature and soil moisture, etc., in which the changes also affected the quality and quantity of the root exudates or rhizodepositions (Marschner *et al.,* 2004; Wenzel *et al.,* 2004; Xu *et al.,* 2009). Generally, maize growth correlates with soil moisture, K, P, OM, and temperature (NSW, 2009). The abiotic factors, especially metals bioavailability in the agriculture soil, were not significantly correlated with the metals accumulation as indicated by the Spearman's rank correlation coefficients in Table 4.4. In the rhizosphere, many parameters, such as period of the field experiment, maize growth, soil properties (pH, Ec, OM, and soil moisture), and rhizobacterial communities were correlated to the bioavailability of the heavy metals. The Shannon-Wiener indeices in the rhizoshere correlated with the maize growth stages and soil moisture. The maize ages mainly caused a shift in the structure of indigenous microbial communities after planting (Piromyou *et al.,* 2011).

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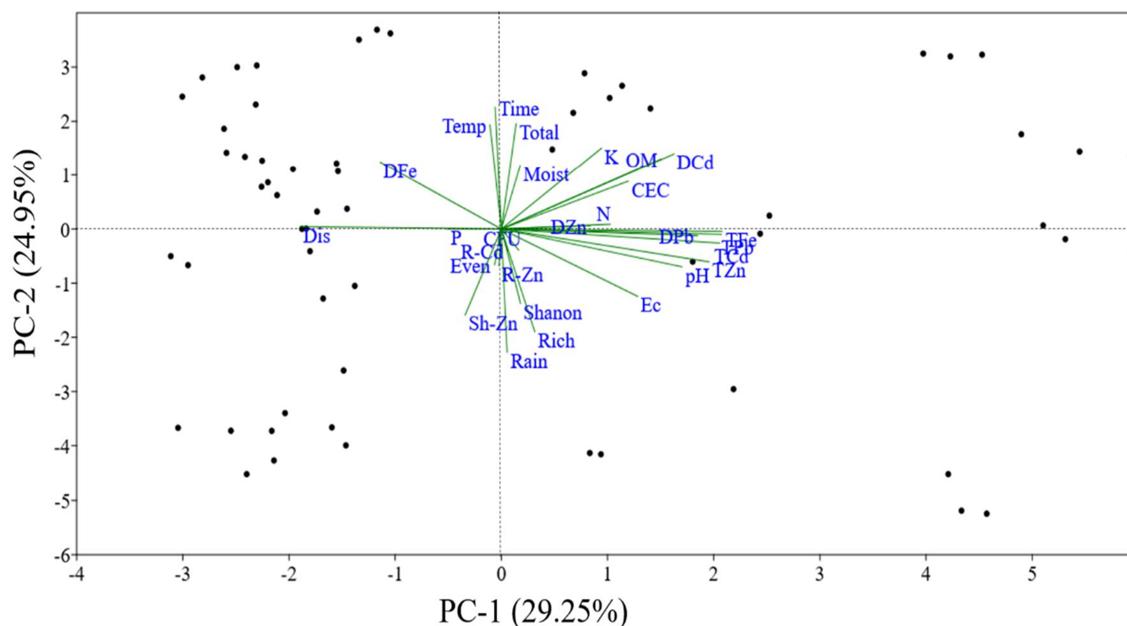

Figure 4.2 Principal component analysis of abiotic factors and bacterial community in rhizospheric soil. (Dis, distances from irrigation source of Mae Tao Creek; Time, maize growth stages; R-Zn, R-Cd and Sh-Zn, metal accumulation in root and shoot; Rain, rainfall; Temp, temperature; Ec, electrical conductivity; Om, organic matter; P, phosphorous; K, potassium; N, nitrogen; CEC, cation exchange capacity; Mois, percentage of soil moisture; TZn, TCd, TPb and TFe, total concentration of metals in soil; DZn, DCd, DPb and DFe, extractable concentration of metals in soil; CFU, number of heterotrophic bacteria; Rich, richness; Even, evenness; Shannon, Shannon-Weiner index; Total, total dry weight of maize) (n=60)

The maize growth stages influenced the microbes in rhizosphere by the flow of low and high molecular weight organic substrates for altering the chemistry of the soil in the vicinity of the plant roots, and by the liberation of selective growth substrates for soil microbes (Brimecombe *et al.,* 2001; Da Mota *et al.,* 2008). Much research has reported the correlation between maize growth stages and microbial community in rhizosphere (Gomes *et al.,* 2001; Baudoin *et al.,* 2002; Da Mota *et al.,* 2008;Cavaglieri *et al.,* 2009). Moreover, the bioavailability of metals were controlled by both external (soil association) and internal (plant association) factors (Sheoran *et al.,* 2016). Metal dynamics were the most intense in the surface soil, because it was characterized by more abundant and

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diverse microbial structure, organic matter content, and cation exchange capacity, etc. (Adriano *et al.*, 2004). Microorganisms controlled the transformation of trace elements by various mechanisms. They included oxidation, reduction, methylation, demethylation, complex formation, and biosorption process (Alexander, 1999). Lynch and Whipps (1991) showed that the mineralogical of the rhizosphere from six different sites of the Unite State were affected by invading the root microorganisms. Xue *et al.,* (2014) showed that maize required high Zn and Fe for optimum maize growth. In addition, plant species, phytoavailability of metals, pH, electrical conductivity, and nutrient were the factors affecting the metal accumulation (Nouri *et al.,* 2009). The phytoavailability of inorganics is usually presented as cations or anions, which are hydrophilic. The bioavailability of cations were inversely correlated with soil CEC (Pilon-Smits, 2005).

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Table 4.4 Spearman's rank correlation coefficient between abiotic factors, biotic factors, rainfall, temperature, and culturable rhizobacterial community with various distances from irrigation source and time of maize growth stages for rhizospheric soil (n=60)

	Distance	Time	Rain	Temp	pH	Ec	Om	P	K	N	CEC	Moisture	TZn	TCd
Distance	1.000	0.000	0.000	0.000	-.796(**)	-.572(**)	-.452(**)	0.229	-.413(**)	-0.245	-.354(**)	-0.005	-.723(**)	-.727(**)
Time		1.000	-1.000(**)	.949(**)	-0.167	-.548(**)	.566(**)	-0.157	.538(**)	-0.007	.265(*)	.420(**)	-.378(**)	-0.078
Rain			1.000	-.949(**)	0.167	.548(**)	-.566(**)	0.157	-.538(**)	0.007	-.265(*)	-.420(**)	.378(**)	0.078
Temp				1.000	-0.084	-.595(**)	.560(**)	-.303(*)	.391(**)	-0.077	0.179	0.240	-.374(**)	-0.136
pH					1.000	.495(**)	.343(**)	-0.172	0.136	0.206	.366(**)	-0.050	.721(**)	.659(**)
Ec						1.000	0.009	0.184	0.143	.321(*)	0.072	-0.020	.651(**)	.639(**)
Om							1.000	-.300(*)	.506(**)	.364(**)	.486(**)	.278(*)	.318(*)	.521(**)
P								1.000	0.171	0.184	0.131	.263(*)	-0.096	-0.011
K									1.000	.296(*)	.293(*)	.613(**)	0.134	.459(**)
N										1.000	.320(*)	.272(*)	.276(*)	.403(**)
CEC											1.000	0.185	.412(**)	.481(**)
Moisture												1.000	-0.114	0.147
TZn													1.000	.815(**)
TCd														1.000
TPb														
TFe														
DZn														
DCd														
DPb														
DFe														
CFU														
Rich														
Even														
Shannon														
TotalDry														
RootZn														
RootCd														
ShootZn														

Superscripts * and ** show significant differences at *P<0.05* and *P<0.01*, respectively

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Table 4.4 (cont’)

	TPb	TFe	DZn	DCd	DPb	DFe	CFU	Rich	Even	Shanon	TotalDry	RootZn	RootCd	ShootZn
Distance	-.685(**)	-.804(**)	0.042	-.520(**)	-.585(**)	.728(**)	-0.065	-0.190	0.015	-0.077	-0.076	0.125	0.045	0.193
Time	-0.073	0.004	-0.011	.547(**)	-0.139	.548(**)	-0.036	-.555(**)	-.258(*)	-.537(**)	.797(**)	-0.008	-0.065	-.474(**)
Rain	0.073	-0.004	0.011	-.547(**)	0.139	-.548(**)	0.036	.555(**)	.258(*)	.537(**)	-.797(**)	0.008	0.065	.474(**)
Temp	-0.142	0.051	0.015	.413(**)	-0.056	.534(**)	0.189	-.419(**)	-.371(**)	-.545(**)	.739(**)	0.157	0.112	-.281(*)
pH	.571(**)	.717(**)	-0.069	.265(*)	.590(**)	-.725(**)	0.224	.289(*)	-0.104	0.094	-0.198	0.066	.272(*)	0.027
Ec	.626(**)	.427(**)	0.057	0.099	.461(**)	-.672(**)	0.011	.403(**)	0.073	.274(*)	-.519(**)	-.303(*)	-0.174	0.162
Om	.557(**)	.623(**)	.344(**)	.736(**)	.525(**)	-0.041	0.156	-0.180	-.263(*)	-.313(*)	.510(**)	0.081	0.095	-.328(*)
P	-0.072	-.368(**)	-.267(*)	-0.106	-.346(**)	0.052	-0.170	-0.025	0.021	0.034	-.322(*)	-.266(*)	-0.228	-0.063
K	.395(**)	0.244	0.080	.669(**)	0.164	0.084	-.278(*)	-0.193	-0.020	-0.180	.384(**)	-0.162	-0.163	-.465(**)
N	.476(**)	.315(*)	.264(*)	.368(**)	.288(*)	-0.129	-0.030	0.046	-0.083	-0.031	-0.114	-0.046	-0.070	0.025
CEC	.457(**)	.488(**)	0.005	.622(**)	.326(*)	-.303(*)	-0.019	-0.193	-0.201	-.274(*)	.289(*)	-.282(*)	-0.113	-.343(**)
Moisture	0.192	-0.039	-0.069	.341(**)	-0.109	.280(*)	-.453(**)	-0.239	0.119	-0.092	0.126	-0.093	0.012	-.449(**)
TZn	.750(**)	.770(**)	.265(*)	.379(**)	.762(**)	-.768(**)	0.108	.312(*)	0.012	0.219	-0.198	-0.132	0.026	0.037
TCd	.900(**)	.766(**)	.325(*)	.655(**)	.740(**)	-.597(**)	0.054	0.097	-0.039	0.061	0.008	-0.180	-0.019	-0.184
TPb	1.000	.765(**)	.346(**)	.627(**)	.661(**)	-.539(**)	0.000	0.086	0.019	0.093	-0.001	-0.193	-0.139	-0.229
TFe		1.000	0.232	.550(**)	.759(**)	-.634(**)	.288(*)	0.163	-0.180	-0.035	0.128	-0.009	0.082	-0.077
DZn			1.000	.284(*)	.470(**)	0.129	0.129	0.059	-0.019	0.091	0.091	0.098	0.046	0.148
DCd				1.000	.465(**)	-0.134	-0.164	-.372(**)	-0.085	-.272(*)	.617(**)	-0.247	-0.246	-.574(**)
DPb					1.000	-.499(**)	.335(**)	0.217	-0.227	-0.030	0.018	0.041	0.156	0.095
DFe						1.000	-0.086	-.375(**)	-0.067	-.255(*)	.336(**)	0.114	0.031	-0.036
CFU							1.000	0.223	-.758(**)	-.423(**)	-0.077	.298(*)	.359(**)	.479(**)
Rich								1.000	0.126	.587(**)	-.599(**)	.297(*)	.341(**)	.440(**)
Even									1.000	.839(**)	-0.095	-0.079	-0.218	-0.234
Shannon										1.000	-.422(**)	0.131	0.025	0.078
TotalDry											1.000	-0.154	-0.234	-.535(**)
RootZn												1.000	.660(**)	.272(*)
RootCd													1.000	.320(*)
ShootZn														1.000

Superscripts * and ** show significant differences at $P<0.05$ and $P<0.01$, respectively

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4.2.4 Identification of culturable bacteria

Seven rhizobacterial isolates that were found in all maize growth stages were selected from their colony morphology (Figure 4.3). The partial 16S rDNA gene of the isolates were amplified by PCR. The partial 16S rDNA sequences were submitted to the GenBank database. The identity values of the bacterial stains varied between 95% and 99%. The phylogeny of the bacterial isolates based on their partial 16S rDNA genes were constructed as shown in Figure 4.4. The sequences were assigned to GenBank accession numbers of KY618802 and KY629622 to KY629627. Three isolates belonged to a as *Bacillus* sp. The colony morphotypes were entire-umbonate-smooth-translucent (KY629623), undulate-raised-smooth-translucent (KY629625), and undulate-raised-wrinkled-translucent (KY629626). Other isolates were closely related to *Brevibacillus agri* (KY618802), *Kocurai rosea* (KY629622), *Cellulosimicrobium funkei* (KY629624), and *Pseudomonas chlororaphis* (KY629627). *Bacillus* species (KY629625) and *Kocurai rosea* (KY629622) appeared at the planting stage of the maize growth. Some bacterial strains were reported as essentially ubiquitous in the agricultural systems (Table 4.5). Many research studies reported that *Bacillus*, *Brevibacillus,* and *Pseudomonas* could promote plant growth and health (Govindasamy *et al.,* 2010; Piromyou *et al.,* 2011; Chauhan *et al.,* 2015). *Pseudomonas chlororaphis* and *Bacillus* sp. could to applied as biological control agents against *Aspergillus fiavus* and *Fusarium verticillioides*, which were widespread inhabitants of agricultural soils associated with many crops including maize (Palumbo *et al.,* 2007; Figueroa-López *et al.,* 2016). In addition, inoculation of *Kocuria rosea* in soil promoted Zn, Cd, and Fe accumulation in sunflower (Mohammadzadeh *et al.,* 2016).

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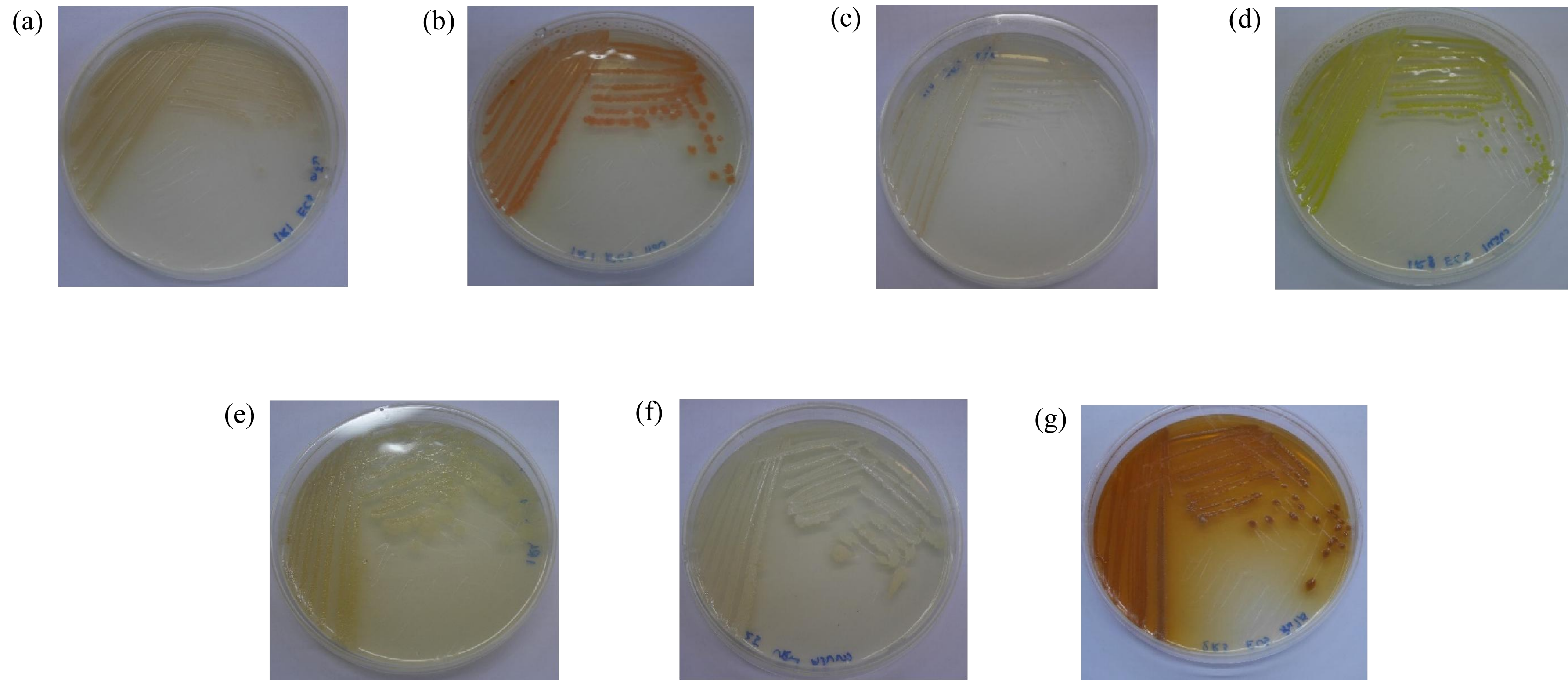

Figure 4.3 Colony morphology of seven culturable bacteria. (a) *Brevibacillus agri* (KY618802), (b) *Kocurai rosea* (KY629622), (c) *Bacillus* sp. (KY629623), (d) *Cellulosimicrobium funkei* (KY629624), (e) *Bacillus* sp. (KY629625), (f) *Bacillus* sp. (KY629626), (g) *Pseudomonas chlororaphis* (KY629627)

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Table 4.5 Roles of bacterial isolates in plant growth

Groups of bacteria	Roles of bacteria	References
Brevibacillus agri	- Phosphate solubilizer	Gene bank. (Acession: JX512031)
	- Plant growth promoting rhizobacteria of *Ocimum sanctum* L.	Singh *et al.,* 2015
Brevibacillus sp.	- Plant growth promoting rhizobacteria of corn in Thailand	Piromyou *et al.,* 2011
Kocurai rosea	- Promoting Zn, Cd, and Fe accumulation in sunflower	Mohammadzadeh *et al.,* 2016
Cellulosimicrobium funkei	- Enhancing growth of *Phaseolus vulgaris* under Chromium(VI) toxicity	Karthik *et al.,* 2016
Pseudomonas chlororaphis	- Biological control agents against *Aspergillus fiavus* and *Fusarium verticillioide*s in maize	Govindasamy *et al.,* 2010
Pseudomonas sp.	- Plant growth promoting bacteria for agriculture	Palumbo *et al.,* 2007
Bacillus sp.	- Plant growth promoting bacteria for agriculture	Govindasamy *et al.,* 2010
	- Biological control agents against *Aspergillus fiavus* and *Fusarium verticillioides* in maize	Palumbo *et al.,* 2007

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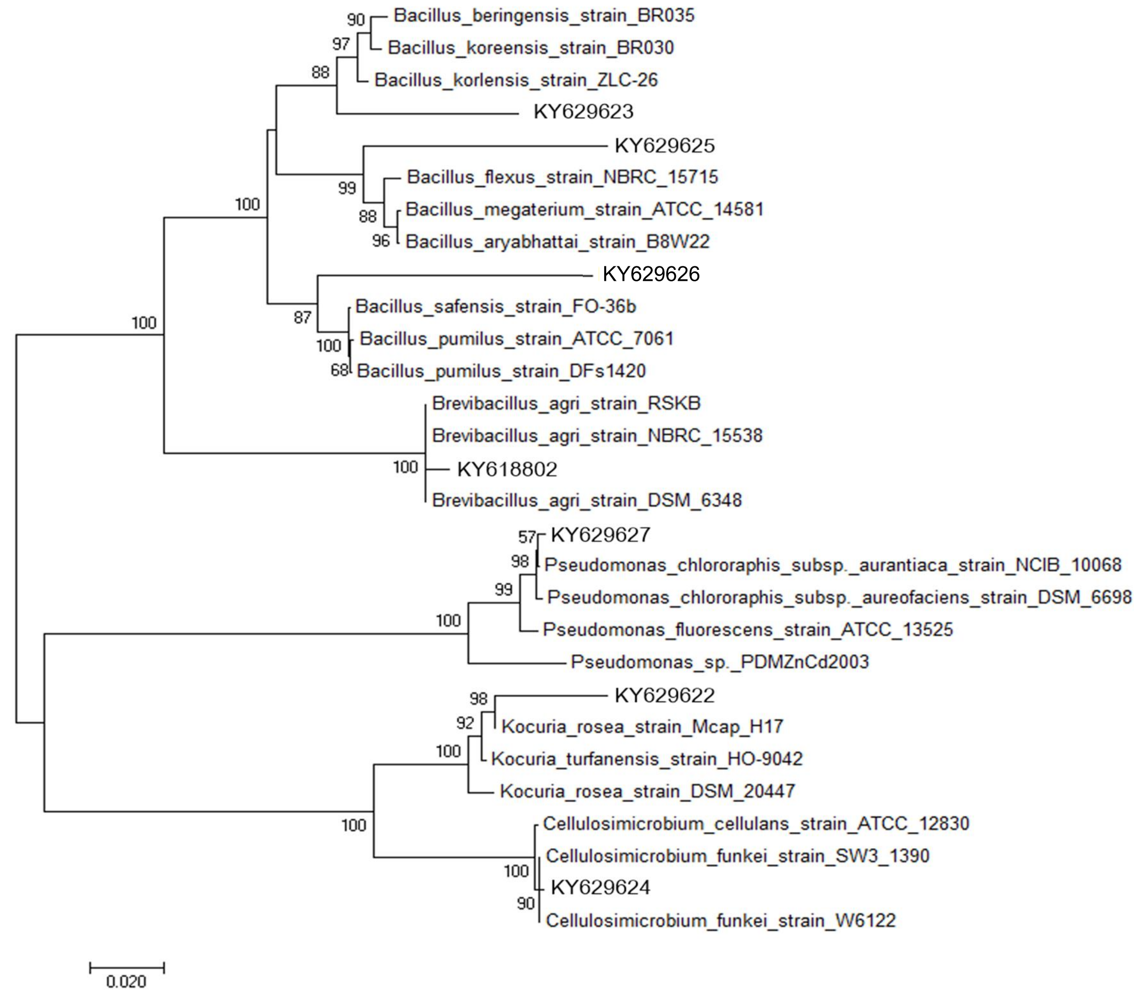

Figure 4.4 Phylogenetic tree of 16S rDNA sequencing for seven bacteria isolated from rhizosphere of maize growing in Zn, Cd, and Pb contaminated soil. This tree was constructed by Neighbor-joining statistical method and Kimura-2-parameter model with 1000 bootstraps.

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4.2.5 PCR-DGGE comparison of bacterial community structure in rhizospheric soil

The bacterial communities in the rhizospheric soils of maize growing in the heavy metals contaminated soils were evaluated by the PCR-DGGE approach as shown in Figure 4.5. The DGGE patterns were obtained from the soil at the VE stage and the rhizospheric soils at V10, R1, R4, and R6 stages of plot numbers 1 and 5, which contained the higtest and the lowest concentrations of the heavy metals, respectively (Appendix H). The PCR-DGGE fingerprints from the higtest and the lowest concentrations of the heavy metals in the rhizospheric soils were significantly different in patterns and density of bands as shown in Figure 4.5(a). A dendrogram of the soil microbes based on the PCR-DGGE bands (Figure 4.5(b)) showed the unculturable and culturable bacterial communities of soils from plots 1 and 5 of the VE stages were seperate from the rhizospheric soils from the other growth stages (Figure 4.5(b)) with a cluster similarity of 62.5%. The second main cluster (67.5% similarity) split the bacterial communities of the rhizospheric soils from V10, R1, R4, and R6 stages into two groups of plot 1 and plot 5. Therefore, the heavy metals in soils and the maize growth stages had impacts on the culturable and unculturable rhizobacterial communities. Espcially, our results indicated that the heavy metals might affect the unculturable bacteria and supported their mild effects of the extracable metals on culturable bacteria (Table 4.4).

Some PCR-DGGE bands, which were found in all stages and in some stages, were collected, subsequently sequenced, and identified as shown in Table 4.6. The V3 region, which is the most suitable for distinguishing all bacterial species to the genus level (Chakravorty *et al.,* 2007), apart from their 16S rRNA genes, was investigated. Unfortunately, the short fragments (150 to 200 bp) of our DGGE products were not sufficient to classify the bacteria into species level (Life technologies, 2013). Therefore, the selected bands were classified into Acidobacteria, Candidatus, α-Proteobacteria, β-Proteobacteria, Firmicutes and Actinobacteria with over 95% similarities. In which, the effect of metals on a decrease in bacterial diversity, increase of Proteobacteria, and decrease of Acidobacteria and Actinobacteria have been reported Gołębiewski *et al.,* (2014).

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(a)

(b)

Figure 4.5 Bacterial community structure from rhizosphere of maize growing in Zn, Cd, and Pb contaminated soil. Dendrogram of genetic similarities of soil microorganism based on PCR-DGGE bands. (a) PCR-DDGE bands and densitometry analysis; (b) dendrogram of soil microorganism based on PCR-DGGE bands. (VE, emergence stage; V10, tenth-leaf stage; R1, silking stage; R4, dough stage; R6, maturity stage; -1, -5, plot nos. 1 and 5)

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Table 4.6 Identification of bands obtained by PCR-DGGE based on V3 region of 16S rDNA and closest sequence match of known bacteria in NCBI database

Band	Taxonomic description	Similarity %	Present in sample (stage of maize)
DGGE-1	Acidobacteria	100	VE, V10, R1
DGGE-2	Candidatus	98	VE
DGGE-3	Proteobacteria	100	VE
DGGE-4	β-Proteobacteria	96	VE
DGGE-5	Firmicutes	96	VE, V10, R1, R4, R6
DGGE-6	α-Proteobacteria	97	VE, V10, R1, R4, R6
DGGE-7	Proteobacteria	99	VE, V10, R1, R4, R6
DGGE-8	Proteobacteria	99	VE, V10, R1, R4, R6
DGGE-9	Firmicutes	99	VE, V10, R1, R4, R6
DGGE-10	α-Proteobacteria	99	V10, R1, R4, R6
DGGE-11	Firmicutes	95	VE, V10, R1, R4, R6
DGGE-12	Actinobacteria	97	V10, R1, R4, R6
DGGE-13	β-Proteobacteria	98	VE, V10, R1, R4, R6
DGGE-14	Acidobacteria Firmicutes Proteobacteria	99	VE, V10, R1, R4
DGGE-15	Actinobacteria	99	VE, V10, R1, R4, R6
DGGE-16	Actinobacteria	100	VE, V10, R1, R4, R6
DGGE-17	α-Proteobacteria	97	VE, V10, R1, R4, R6
DGGE-18	Acidobacteria Firmicutes	99	VE, V10, R1, R4, R6
DGGE-19	Actinobacteria Firmicutes	99	VE, V10, R1, R4, R6

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Proteobacteria, Actinobacteria, Firmicutes, Acidobacteria, Cadidatus, and Bacteroidetes have been found as rhizobacteria colonizers on maize roots. In addition, the dominant genera rhizobacteria of maize were *Bacillus*, *Ralstonia, Sphingobium/Sphingomonas*, *Streptomyces, Rhodococcus, Cupriavidas, Pseudomonas, Bradyrhizobium, Agrobacterium/Rhizobium* etc. (Estrada-de los Santos *et al.,* 2011; García-Salamanca *et al.,* 2013; Peiffer *et al.,* 2013; Qaisrani *et al.,* 2014; Correa-Galeote *et al.,* 2016; Johnston-Monje *et al.,* 2016). These bacteria are usually associated with agricultural crops. Therefore, some bacterial isolates probably improve the agricultural ecosystems and environment sustainability in the maize field.

4.2.6 Maize growth and accumulation of metals in shoot parts

The effects of heavy metals on maize growth were determined by height and total dry weight (Appendix I). The results showed a slight effect of the heavy metals on plant growth. Height of maize was elongated from V10 (30 days) to R1 stage (60 days), and was slightly elongated until R6 stage (60-120 days). The averages of total dry weight and height of maize in the harvesting stage (R6) of these results were 224.1±44.2 g of dry weight and 223.8±25.1 cm of shoot height. Our maize growth was similar to the dry biomass and heights of maize growing in uncontaminated soil as 258.0±37.8 g and 175.3±23.7 cm, respectively (Cheng *et al.,* 2015). During maize development, groundcover lies on top of the soil (Appendix C), such as Siam weed, it might help phytoremediation of the heavy metals. Siam weed was a native plant found in the Zn mining (Prasad *et al.,* 2015), and the weed accumulated Cd, especially in the aboveground parts (Sampanpanish *et al.,* 2008).

Effects of the heavy metals and the maize growth stages on metals accumulation in the root and shoot (Appendix J) showed that the contaminations and the growth stages significantly affected the Zn and Cd accumulation ($P<0.01$). Accumulation of Pb in maize was under the limit of detection for our analytical system. *Translocation factor (TF)* (Baker *et al.,* 1990) and *Biological Absorption Coefficient* (*BAC*) (Ferguson, 1990) were determined following equations (3) and (4), respectively.

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$$TF = \frac{concentration\ of\ metals\ in\ shoot\ (mg/kgdry\ wt.)}{concentration\ of\ metals\ in\ root\ (mg/kg\ dry\ wt.)} \quad (3)$$

$$BAC = \frac{concentration\ of\ metals\ in\ root\ (mg/kg\ dry\ wt.)}{Total\ concentration\ of\ metals\ in\ soil\ (mg/kg\ dry\ wt.)} \quad (4)$$

TF values for Zn greater than 1.0 indicated that the maize was a Zn hyperaccumulator (Table 4.7, Appendix K). The highest percentages of Zn contents in the phytomass were obtained from the maize growing in plot 1, whereas the lowest Zn contents were from the maize in plot 5. Figure 4.6 shows that 50 percent of the Zn accumulation were transported to shoot parts, especially with maize growth in high Zn contaminated soil. The Zn concentrations in seeds were increased from 11 mg kg^{-1} in plot 1 to 30 mg kg^{-1} of dry weight in plot 5 (Table 4.7). The Cd was mainly accumulated in the roots and Cd accumulation in the shoot could only be detected at the V10 stage of maize development. *BAC* has been applied currently to evaluate Cd root absorption. The result showed that Cd was easily absorbed by roots (range 0.2-2.6) (Table 4.7).

Zn was one of the trace elements for proper maize growth (NSW, 2009). In addition, Zn rich maize seeds could be produced from Mae Sot for feed. Sozubek *et al.,* (2014) showed that Zn contents of roots were higher than shoots when grown in unpolluted soil, and Zn was generally greater in shoots than the roots of maize grown in polluted soil with Cd. The concentrations of metals in the maize increased as follows: Zn>Ni>Pb by Pb and Ni mainly accumulation in the maize roots, and Zn mainly accumulation in the maize fruit (Lu *et al.,* 2015). The high performance of the maize root for Cd absorption was a mechanism to prevent/reduce deleterious effects of the Cd on carbon assimilating apparatus of the aerial parts of the plant restricted Cd in the roots (Moreira *et al.,* 2014). Plant cells can counteract the toxicity of Cd in several ways and the cell wall actively participates in most of these mechanisms. For example, specific polysaccharides, proteins, cell wall phosphates, and secondary modifications of the cell wall, such as lignin, restricted Cd on the cell wall (Parrotta *et al.,* 2015). The metal can be immobilized in the cellular walls and intercellular spaces, which is restricted in the root tip and to the regions of lateral roots initiate (Lux *et al.,* 2011). On the other hand, the higher mobility of Zn than Cd caused Zn transport over long distances in maize, and Cd stayed in the roots abundantly (Sozubek *et al.,* 2014). Cd uptake involved the ZNT1

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transporter, which was shown to mediate a high affinity Zn and low affinity Cd (Pence *et al.,* 2000). The antagonistic effect of Zn on Cd accumulated in plant tissues was by decreasing Cd accumulation in the crop (Nan *et al.,* 2002). Zhang *et al.,* (2008) reported Cd uptake by maize in the mature stage had a significant genetic variation, and 70-85% of total absorbed Cd was stored in the roots. In addition, the results in Table 4.7 support phytoextraction by growing maize. High Zn concentrations in the edible maize seeds, with a limit for Cd, were interesting for supply as animal feed. Maize was one of the most susceptible cereal crops to Zn deficiency (Bouis and Welch, 2010). One global challenge from agriculture was to increase grain micronutrient (such as Zn and Fe) concentrations in main cereal crops (Zhang *et al.,* 2013). Maize seed-Zn levels ranged from 16.5 to 24.6 mg kg^{-1} of dry weight (Cortez and Ching, 2014).

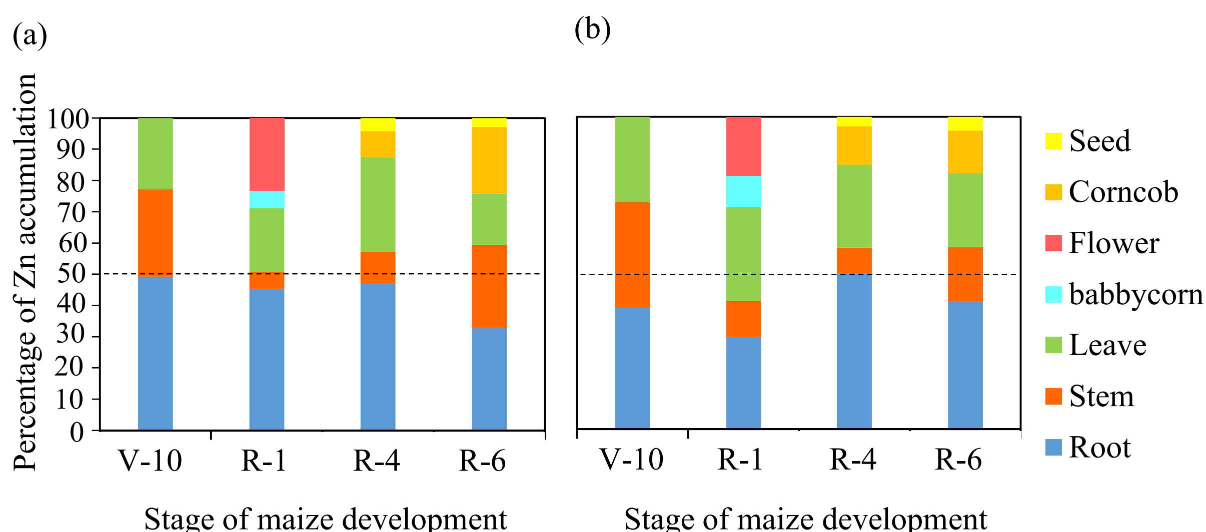

Figure 4.6 Percentages of Zn content in parts of maize (root, stem, leaves, baby corn, flower, corncob, and seed) at growth stages of V10, R-1,-4, and -6; (a) plot 1, (b) plot 5 (n=3)

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Table 4.7 *Translocation factor (TF)* and *biological absorption coefficient (BAC)* value of maize and accumulation of Zn in maize seed

Accumulation parameters	Value	References
Translocation factor (TF)	0.59-2.62	*TF*>1*
Biological Absorption Coefficient (BAC)		
BAC of Root-Zn	0.06-0.65	0.22-0.90[a]
BAC of Root-Cd	0.20-2.08	0.22-0.90[a]
Zn concentration in seed (mg kg^{-1} dry wt)	11.19-30.22	16.5-24.6[b]

* TF> 1 is hyperaccumulator (Baker *et al.,* 1990), [a] general value of *BAC* (Wahsha *et al.,* 2014), [b] general value of metals concentration in seed (Cortez and Ching, 2014).

4.2.7 Assessment of soil contamination levels and ecological risk

The degree of heavy-metal contamination was determined by the *pollution index* (C_f) and *potential ecological risk index* (E_f) for the three heavy metals of Zn, Cd, and Pb, which were distributed in the field-grown maize in Mae Sot. The C_f and E_f were defined as in equations (5) and (6), respectively (Guo *et al.,* 2010):

$$C_f = \frac{Ci}{Si} \qquad (5)$$

$$E_f = C_f \times T_f \qquad (6)$$

Where Ci is the measured concentration of the examined metals in the soils and Si is the geochemical background concentration of the metals. Thai background values (mg kg^{-1}) utilized were 0.17 for Cd, 54.6 for Pb, and 71 for Zn (Zarcinas *et al.,* 2004). T_f is the biological toxic factor of a single element, which was determined as 1 for Zn, 5 for Pb, and 30 for Cd (Hakanson, 1980).

Table 4.8 shows that the *pollution index* (C_f) and *potential ecological risk index* (E_f) calculated according to the natural background values of heavy metals in soils of Mae Sot varied considerably across the different metals contaminated soil and different maize plant growth stages with planting and ripening stage. The C_f values ranged from 4.26 to 43.99 for Zn, 32.67 to 388.59 for Cd, and 0.55 to 2.51 for Pb. The Cd contaminated

soils had the highest *pollution index*. The *potential ecological risk factor* of heavy metals in the field-grown maize in Mae Sot were Cd>Zn>Pb; Cd is the most important one for risk factor. The E_f values ranged from 4.26 to 43.99 for Zn, 980.00 to 11,657.65 for Cd, and 2.76 to 12.53 for Pb. The C_f and E_f values for all metals (Zn, Cd, and Pb) indicated that field-grown maize in Mae Sot was seriously contaminated soil, especially in the agricultural area near the irrigation source of Mae Tao Creek. However, sowing maize in this area could remediate the soil by gradually reducing C_f and E_f values.

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Table 4.8 *Pollution index* (C_f) and *potential ecological risk index* (E_f) of heavy metals in rhizosphere of maize at planting stage and ripening stage (n=3)

Plots	*Pollution index* (C_f)						Risk grade (Sun *et al.,* 2010)
	Zn		Cd		Pb		
	Planting	Ripening	Planting	Ripening	Planting	Ripening	
1	43.99 ± 2.13	30.76 ± 0.36	388.59 ± 17.30	217.73 ± 7.41	2.51 ± 0.07	1.87 ± 0.01	
2	24.26 ± 1.37	16.33 ± 0.55	163.84 ± 16.81	94.65 ± 13.55	1.39 ± 0.09	1.06 ± 0.18	Low: C_f<1
3	12.64 ± 0.94	10.49 ± 1.13	61.78 ± 1.40	52.16 ± 11.25	0.82 ± 0.07	0.72 ± 0.21	Moderate: 1< C_f<3
4	11.59 ±1.20	8.89 ± 1.47	48.16 ± 5.32	71.25 ± 10.41	0.78 ± 0.14	0.77 ± 0.04	High: C_f>3
5	4.26 ± 1.49	14.59 ± 1.32	32.67 ± 5.74	90.45 ± 6.79	0.55 ± 0.02	0.95 ± 0.13	

Plots	*Potential ecological risk index* (E_f)						Risk grade (Hakanson, 1980)
	Zn		Cd		Pb		
	Planting	Ripening	Planting	Ripening	Planting	Ripening	
1	43.99 ± 2.1	30.76 ± 0.36	11 ,657.65 ± 519.05	6,531.76 ± 222.22	12.53 ± 0.36	9.37 ± 0.06	Low: <40
2	24.26 ± 1.4	16.33 ± 0.55	4,915.29 ± 504.26	2,839.41 ± 406.52	6.96 ± 0.47	5.30 ± 0.91	Moderate: 40-80
3	12.64 ± 0.9	10.49 ± 1.13	1,853.53 ± 41.92	1,564.71 ± 337.56	4.08 ± 0.37	3.59 ± 1.04	Considerable: 80-160
4	11.59 ± 1.2	8.89 ± 1.47	1,444.71 ± 159.53	2,137.65 ± 312.38	3.90 ± 0.69	3.86 ± 0.21	High: 160-320
5	4.26 ± 1.5	14.59 ± 1.32	980.00 ± 172.22	2,713.53 ± 203.79	2.76 ± 0.11	4.75 ± 0.65	Significantly high: >320

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4.3 Pot experiment

The results obtained from the field experiment indicated that many parameters of the rhizosphere were highly correlated to the maize growth stages. Culturable rhizobacterial communities were stimulated during the early stage of maize growth for 30 days (V10). In addition, Cd accumulation in shoots was found only in the early stage of maize growth. Our review found that root exudates, such as phenolic compounds and organic acids, were related to the mechanism of plant tolerance stress, metals accumulation, altering the chemistry of the soil, and microbial community. Therefore, maize developments in the four weeks after planting coupled with root exudates were investigated. In the study of the root exudates in the field experiment it was difficult to collect and to preserve the samples. Therefore, a pot experiment was carried out to study the interaction of maize with the three levels of heavy metals (Cd, Zn, and Pb) contaminated in soil, metals accumulation, and root exudates.

4.3.1 Levels of soil contamination

Three levels of heavy metals contaminated soil (Zn/Cd/Pb) were collected from the row spacing of maize growth in plot numbers 1, 3, and 5 of the field experiment (Table 3.4). The amounts of CHNO/S in the soil were enough for maize growth (Barber, 1995). The total concentrations of Zn, Cd, and Pb in the soils were 695-3,200 mg Zn kg^{-1}, 8-57 mg Cd kg^{-1}, and 34-121 mg Pb kg^{-1}. Non-contaminated soil had similar soil properties to the contaminated soil, except for the heavy metal contamination. In addition, the extent of the soil contamination was evaluated by comparing the total concentration of the trace metals in the soils from this study area with Thai investigations on the level of Zn, Cd, and Pb in contaminated agriculture soil (77 mg Zn kg^{-1}, 0.17 mg Cd kg^{-1}, and 55 mg Pb kg^{-1}) (Zarcinas *et al.,* 2004), which suggested moderate to high concentrations of the heavy metals in the contamination soil.

4.3.2 Effects of heavy metals on maize growth

The effects of Zn/Cd/Pb contaminated soil on the growth of maize after 1, 2, 3, and 4 weeks were determined by height, shoot dry weight, and total chlorophyll content. Table 4.9 shows that higher Zn/Cd/Pb contaminated soil resulted in a lower height and dry weight, especially in the early stage of maize growth (1 week). An increase in the growth period tended to decrease the total chlorophyll content, whereas a higher

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Zn/Cd/Pb concentration in the soil tended to promote chlorophyll content when compared with the control plants. The maize had 100% survival and showed no toxicity symptoms throughout the experiment. Spearman's rank correlation coefficient (Table 4.10) shows Zn/Cd/Pb contaminated soils were not significantly correlated with maize growth. Dry weight and height of maize were positively correlated only with period of maize growth ($r=0.959$ and 0.853, respectively). Total chlorophyll contents were correlated with both Zn/Cd/Pb contaminated soils and period of maize growth. There was a positive correlation between Zn/Cd/Pb contaminated soils and upper chlorophyll content ($r=0.336$), whereas the period of maize growth showed a negative correlation with the lower-upper chlorophyll content ($r=-0.479$ and $r=-0.805$, respectively). The results indicated that the metals contaminated soils had a low effect on the maize growth, but changed the chlorophyll content. They were related to the mechanism of heavy metals tolerance. The result obtained in the field showed maize had the ability to tolerate heavy metals in the contaminated soil. This result supported the slight effect of heavy metals on maize growth.

The heavy metals contaminated soil did not affect maize growth and chlorophyll contents, when compared with the maize growing in the control soil. This result supported the results of the filed study, and they implied that the maize had the ability to tolerate the Zn, Cd, and Pb in the contaminated soil. Growing maize in contaminated soil increased the metals available. A key element of acquisition of nutritional metals was the release of exudates with chelator properties from the roots into the rhizosphere, which can select growth substrates for soil microorganisms. One related process concerns the role of root exudates in metal tolerance by metal chelators (Hall, 2002). On the one hand, it can be used as a defense strategy producing less soluble metal complexes that are unsuitable for entering the plant (Viehweger, 2014). Therefore, root exudates were investigated.

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Table 4.9 Effects of Zn/Cd/Pb contaminated soil on maize growth in pot experiment

Parameter	Treatments	Duration of study			
		1 week	2 weeks	3 weeks	4 weeks
Height (cm)					
	CT	$40.33\pm0.58^{C,a}$	$50.00\pm0.00^{B,b}$	$60.00\pm1.73^{A,a}$	$61.7\pm0.58^{A,a}$
	Low	$37.33\pm0.58^{A,ab}$	$52.67\pm0.58^{B,a}$	$59.33\pm1.15^{A,a}$	$53.7\pm0.58^{B,a}$
	Medium	$35.00\pm1.00^{C,ab}$	$52.33\pm0.58^{B,a}$	$58.67\pm0.58^{A,a}$	$58.0\pm1.00^{A,b}$
	High	$32.33\pm2.08^{C,c}$	$53.33\pm0.58^{B,a}$	$59.33\pm0.58^{A,a}$	$59.7\pm0.58^{A,b}$
Shoot dry weight (g)					
	CT	$0.142\pm0.009^{C,a}$	$0.283\pm0.015^{B,a}$	$0.499\pm0.003^{A,a}$	$0.523\pm0.061^{A,a}$
	Low	$0.118\pm0.001^{D,b}$	$0.274\pm0.031^{C,a}$	$0.363\pm0.012^{B,c}$	$0.632\pm0.028^{A,a}$
	Medium	$0.102\pm0.005^{D,bc}$	$0.272\pm0.002^{C,a}$	$0.420\pm0.016^{B,b}$	$0.548\pm0.042^{A,a}$
	High	$0.087\pm0.007^{C,c}$	$0.245\pm0.014^{B,a}$	$0.475\pm0.025^{A,a}$	$0.544\pm0.041^{A,a}$
Upper-chlorophyll content (SPAD unit)					
	CT	$32.53\pm2.34^{A,a}$	$24.63\pm1.17^{B,b}$	$19.17\pm0.46^{C,c}$	$17.57\pm0.12^{C,c}$
	Low	$34.80\pm0.92^{A,a}$	$25.87\pm0.87^{C,b}$	$28.23\pm1.01^{B,a}$	$23.30\pm0.20^{D,b}$
	Medium	$34.90\pm0.26^{A,a}$	$28.90\pm0.36^{B,a}$	$22.70\pm0.26^{D,b}$	$25.40\pm0.26^{C,a}$
	High	$35.03\pm3.67^{A,a}$	$30.83\pm0.81^{AB,a}$	$27.67\pm0.49^{BC,a}$	$23.77\pm0.71^{C,b}$
Lower-chlorophyll content (SPAD unit)					
	CT	$25.60\pm0.40^{A,b}$	$13.27\pm0.93^{C,b}$	$18.93\pm1.25^{B,a}$	$12.87\pm0.92^{C,b}$
	Low	$32.93\pm0.81^{A,a}$	$20.77\pm1.59^{B,a}$	$22.43\pm1.35^{B,a}$	$21.07\pm1.44^{B,a}$
	Medium	$25.63\pm1.71^{A,b}$	$15.43\pm0.61^{C,ab}$	$20.03\pm1.33^{B,a}$	$20.03\pm0.12^{B,a}$
	High	$22.30\pm1.81^{A,b}$	$17.40\pm3.98^{A,ab}$	$21.50\pm1.01^{A,a}$	$20.07\pm0.64^{A,a}$

The different letters (s) (A-D) and (a-c) are significant differences according to Duncan's new multiple range test (*$P<0.01$*) for which (A-D) showed differences in the same duration in each treatment, whereas (a-c) showed differences in each treatment under the same time condition. The data are given as the means±SD (n=3)

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Table 4.10 Spearman's rank correlation coefficient between chlorophyll content, shoot dry weight and height, time of maize growth stages, and treatment with three levels (low, medium, and high) of Zn/Cd/Pb concentrations

	Treatment	Time	Lower chlorophyll	Upper chlorophyll	Shoot dry weight	Height
Treatments	1.000	<.001	.126	.336*	-.112	-.053
Time		1.000	-.479**	-.805**	.959**	.853**
L-Chlorophyll			1.000	.636**	-.441**	-.430**
U-Chlorophyll				1.000	-.814**	-.745**
Shoot-Dry					1.000	.812**
Height						1.000

*, ** significant level at $P<0.05$ and $P<0.01$. Treatments = various Zn/Cd/Pb concentrations, Time = period of maize growth

4.3.3 Metals accumulation in shoot and water extraction of metals

Figure 4.7 (a) and (c) show Zn and Cd concentrations in shoots resulting from various level of Zn/Cd/Pb concentration and period of maize growth. The maize accumulated Zn higher than Cd. The amount of Pb in the shoot could not be detected due to the limit of detection. The Zn concentration in the shoot increased during two weeks. The Cd concentration in the shoot tended to decease when there was an increase in the period of maize growth, whereas Cd was not accumulated in the shoot of maize with the control due to this soil not being Cd contaminated. However, the total Zn and Cd contents per plant tended to increase (Figure 4.7 (b) and (d)). This result indicated that maize transported Zn to shoot parts but prevented Cd transport to the shoot. Decreasing the Zn and Cd accumulation during weeks 3-4 caused slight maize growth that had a diminished absorption area in the plant. Figure 4.8 shows only Zn concentrations in the water extracts, because Cd and Pb were less than the detection limit for the analysis. The results of the water extraction indicated that the growth of maize supported Zn bioavailability in the soil. The mechanism of metal availability and soluble nutrients involved with root exudates have been explained (Carvalhais *et al.,* 2010; Wenzel *et al.,* 2004).

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Spearman's rank correlation coefficients revealed that Zn and Cd concentrations in the shoot were positively correlated with the concentrations of Zn/Cd/Pb in the soil and water soluble Zn, whereas the period and maize growth were Treatments were various Zn/Cd/Pb concentrations in soil, time, duration of negatively correlated (Table 4.11). The correlation obtained from the pot experiment was different in the field experiment. It was caused from the environment, stage of maize growth, and small size of the pot experiment. maize growth, Acc-Zn, Zn accumulation in shoot, Acc-Cd, Cd accumulation in shoot, and Water-Zn water extraction Zn in soil.

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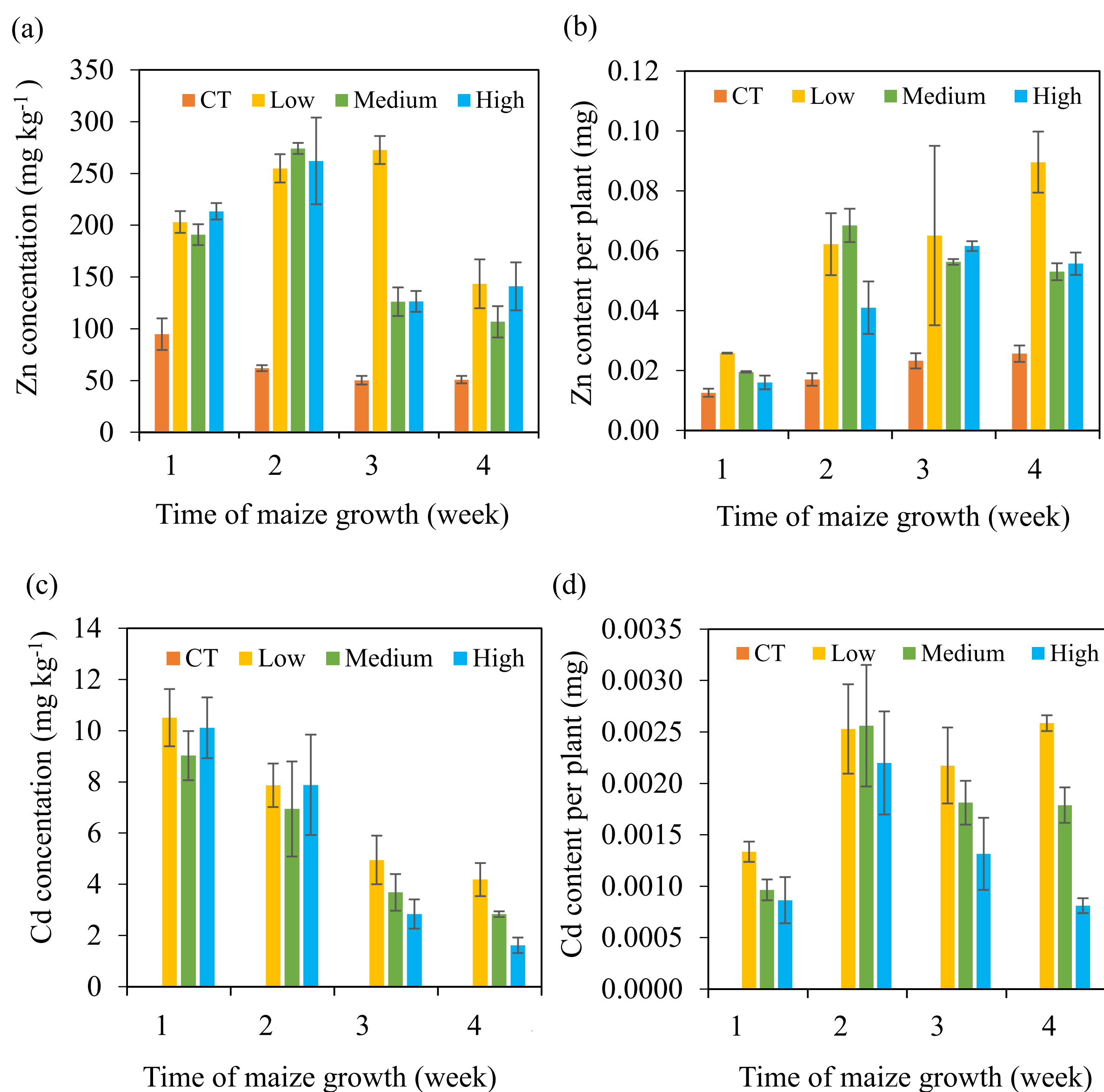

Figure 4.7 Zn and Cd concentrations and amounts of Zn and Cd accumulated per plant in shoots of maize in weeks 1 to 4. Plants were grown in three levels of Zn/Cd/Pb contaminated soil (low, medium, and high) and non-contaminated soil (control). (a) Zn concentration, (b) Zn content per plant, (c) Cd concentration, and (d) Cd content per plant (n=3)

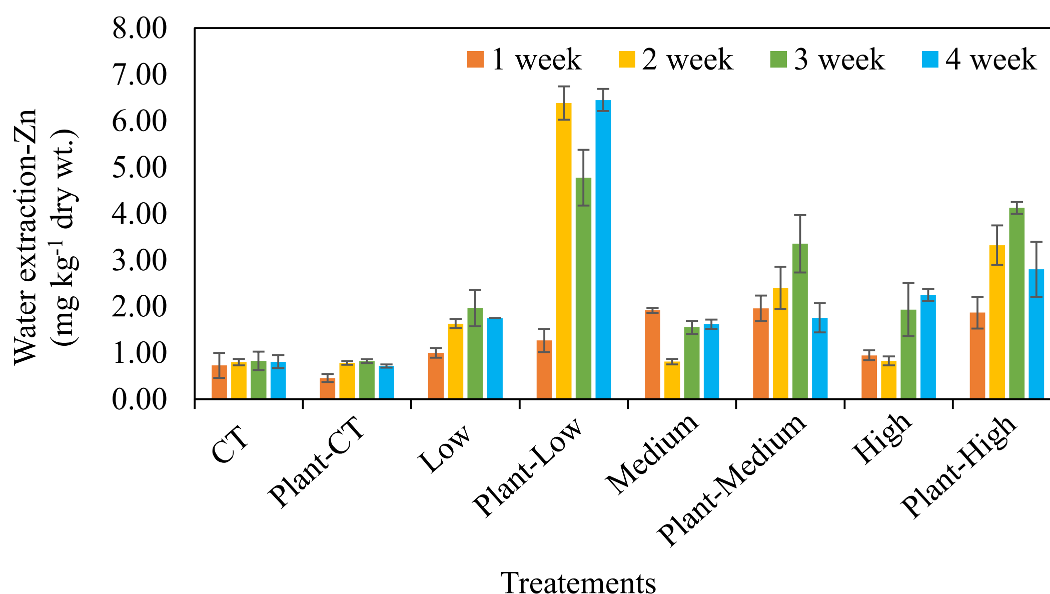

Figure 4.8 Zn concentrations in water extracts of soils, planted, and not-planted maize in weeks 1 to 4. Plants grew in three levels of Zn/Cd/Pb contaminated soil (low, medium, and high) and non-contaminated soil (control). Data are given as means±SD (n=3)

Table 4.11 Spearman's rank correlation coefficients between metal accumulation, shoot dry weight and height, time of maize growth stages, and treatment with three levels (low, medium, and high) of Zn/Cd/Pb concentrations (n=48)

	Treatment	Time	Height	Shoot-Dry	Acc-Zn	Acc-Cd	Water-Zn	TPC	TFC
Treatments	1.000	<0.001	-.053	-.112	.487**	.415**	.480**	-.175	-.108
Times		1.000	.853**	.959**	-.375**	-.544**	.266	-.632**	-.494**
Height			1.000	.812**	-.352*	-.583**	.183	-.688**	-.508**
Shoot-Dry				1.000	-.457**	-.613**	.215	-.617**	-.520**
Acc-Zn					1.000	.814**	.595**	.327*	.383**
Acc-Cd						1.000	.375**	.403**	.330*
Water-Zn							1.000	-.186	-.030
TPC								1.000	.819**
TFC									1.000

The superscripts * and ** show significant differences at $P<0.05$ and $P<0.01$, respectively.

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4.3.4 Total phenolic content and total flavonoid content

The TPC and TFC of root extracts from maize growing in non-contaminated soil (control) and three levels of Zn/Cd/Pb contaminated soil (low, medium, and high) are shown in Figure 4.9(a-b). The box-plots of TPC and TFC in each treatment are shown in Figure 4.10(a-b). The TPC and TFC of the root extracts significantly decreased in the maize growing with the control and the high metals contaminated soil ($P<0.01$). While, the TPC and TFC of the plants treated with different heavy metals tended to slightly increase when compared with the control. The results indicated the changes in the TPC and TFC were related to the heavy metals stress on maize.

Table 4.11 presents Spearman's rank correlation coefficient between metal accumulation, shoot dry weight and height, time of maize growth stages, and treatment with three levels (low, medium, and high) of Zn/Cd/Pb concentrations. The TPC and TFC were negatively correlated with maize growth and period of maize growth, but not correlated with Zn/Cd/Pb contaminated soil. This was different from the previous results, which were obtained by box plot (Figure 4.10) because the Spearman's rank correlation coefficients were based on linear correlation. However, TPC had a higher positive correlation with Cd accumulation in the shoot than TFC ($r=0.403$, $P<0.01$ and $r=0.330$, $P<0.05$, respectively). Whereas, the TFC had a higher positive correlation with Zn accumulation in the shoot than TPC ($r=0.383$, $P<0.01$ and $r=0.327$, $P<0.05$, respectively). In addition, TPC and TFC were not correlated with Zn in the water extracts. This result indicated that the TPC and TFC in the root extract responded in specific ways to different heavy metals accumulation in shoots of maize and related maize growth, which caused heavy metals tolerance of maize and supported heavy metals accumulation in maize.

Phenolic compounds, especially a subgroup of them, such as flavonoid compounds, have been reported a plant secondary metabolites. Which are important in the mechanisms of plants to a plethora of abiotic stress (Di Ferdinando *et al.,* 2012). Since many flavonoids and other phenolic compounds are strong antioxidants, their accumulation in plants can reduce oxidative damage induced by different abiotic stresses, including drought, salinity, and heavy metals stress (Di Ferdinando *et al.,* 2012; Cicevan *et al.,* 2016). Changes in phenolic compounds should be affect by the amount of metal accumulated in the tissue, which can function as metal chelators, and are able to act as

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radical scavengers (Sgherri *et al.,* 2003; Kovacik *et al.,* 2009). Generally, heavy metals stress induced increases in the accumulation of phenolic compounds and flavonoid compounds, such as Cd, and caused an accumulation of soluble phenolics in the cytosol of *Pinus sylvestris* root cells (Schutzendubel *et al.,* 2001). However, the decreasing TPC and TFC contents and the low correlation between TPC, TFC, and Zn/Cd/Pb contaminated soil, was caused by phenolic metabolism. It related to the enzymes that preferentially affected the roots. Sgherri *et al.,* (2003) reported that lipid peroxidation increased and glutathione was oxidized as the copper concentration increased. Di Ferdinando *et al.,* (2012) found that the accumulation of polyphenols was inversely related to the activities of key antioxidant enzymes, which declined steeply as Cd stress progressed.

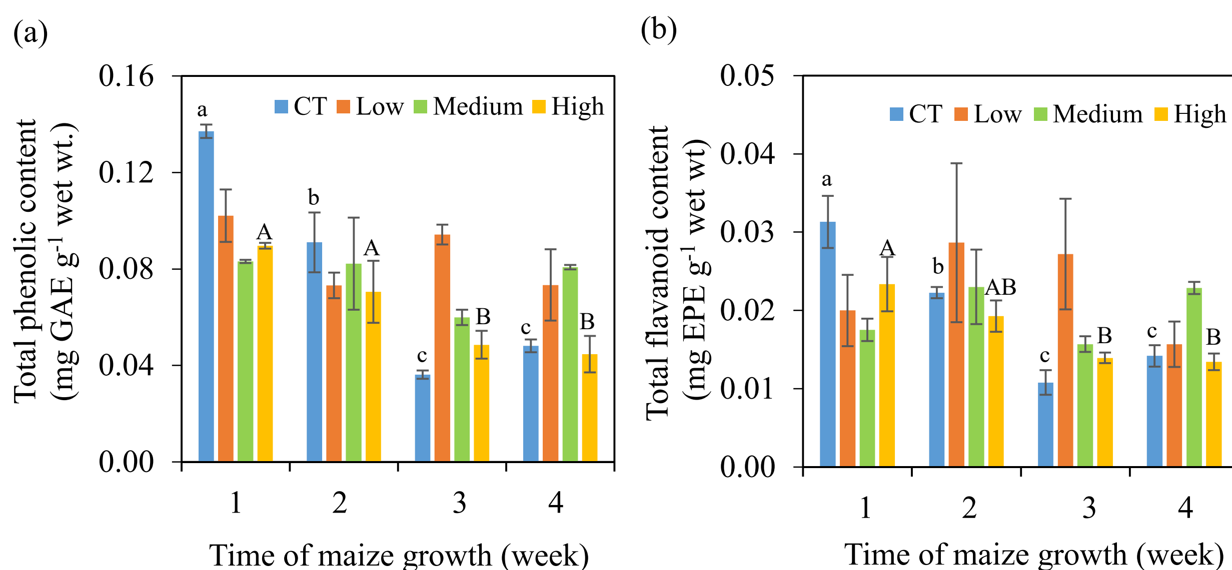

Figure 4.9 Total phenolic content (TPC) and total flavonoid content (TFC) in root extracts of maize in weeks 1 to 4. Plants grew in non-contaminated soil (control) and three levels of Zn/Cd/Pb contaminated soil (low, medium, and high). Different letters (a-c), (A-B) are significant differences according to Duncan's new multiple range test ($P<0.01$). (a) total phenolic content and (b) total flavonoid content (n=3)

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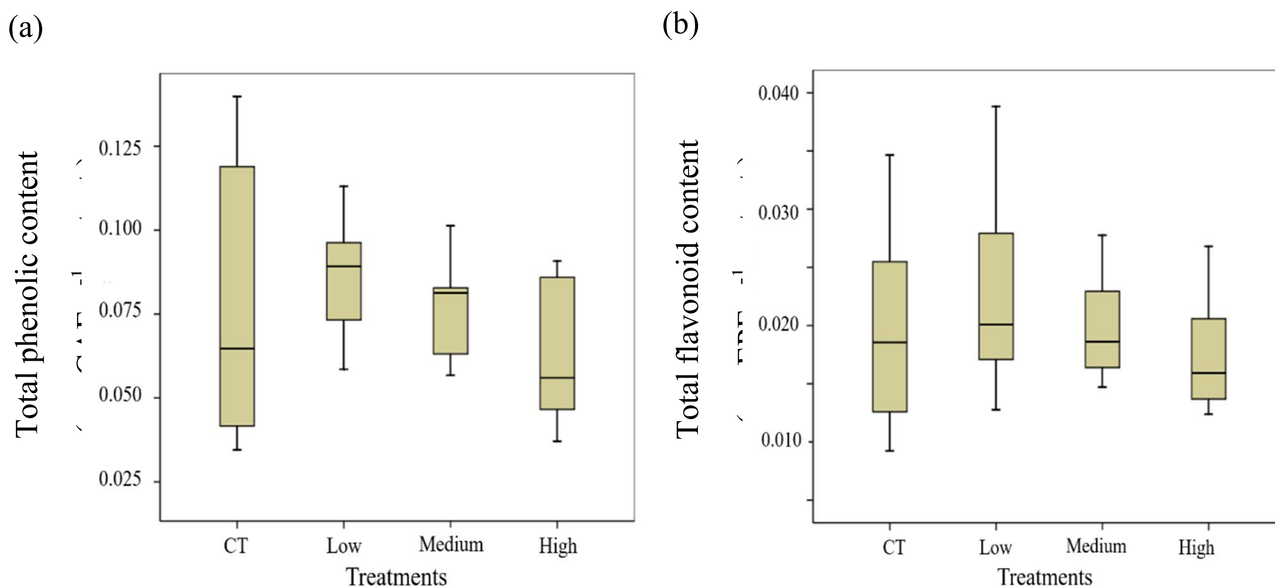

Figure 4.10 Box-plot of total phenolic content (TPC) and total flavonoid content (TFC) in root extracts of maize in weeks 1 to 4. Plants grew in non-contaminated soil (control) and three levels of Zn/Cd/Pb contaminated soil (low, medium, and high). (a) TPC, (b) TFC (n=12)

4.3.5 Analysis of root exudates by HPLC

The previous results showed that the TPC and TFC of root extracts were the important factor for maize growth and metals accumulation. However, many type of phenolic compound and flavonoid compound were related to the mechanism of maize growth, metals tolerance, metals accumulation, and microbial community. In addition, organic acids were root extracts, which were related mainly to the plant under many stresses. Therefore, the identification of these root extracts was investigated.

For the phenolic compounds, 13 phenolic and flavonoid compounds (gallic acid, quercetin, myricetin, catechin, kaempferol, epicatechin, caffeic acid, vanillin, naringenin, chlorogenic, wedelolactone, p-coumalic, and rutin) were used as standard chemicals. Root extracts were identified by comparing the retention times with standard samples. Figure 4.11 shows the HPLC chromatograms of the root extracts obtained from the maize growing in the control and the four treatments for one week. The treatments showed high total TPC and TFC contents (Figure 4.9). The results showed that the four main peak positions the HPLC chromatograms exhibited corresponded to standard

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patterns and retention times of gallic acid, catechin, vanillin, and p-coumalic. Unclear retention times with caffeic acid and epicatechin can be achieved by LC-ESI-QTOF-MS/MS. The concentration of gallic acid, catechin, vanillin, and p-coumalic in the root extracts were calculated by peak height as units of mg standard per wet weight of plant root. The total concentrations of gallic acid, catechin, vanillin, and p-coumalic in the root extract of maize grown in non-contaminated soil and the three levels of Zn/Cd/Pb contaminated soil are shown in Appendix L. The box-plot of the phenolic compounds in the root extracted showed gallic acid, vanillin, and p-coumalic acid contents in the root extraction that were significantly decreased in all of the samples ($P<0.01$), but catechin content was not different in the root extracts after the maize growth ($P>0.01$) (Figure 4.12). The Zn/Cd/Pb contaminated soils caused decreasing catechin content in the root extracts when compared with the control (Figure 4.13). These result supported the TPC and TFC levels in the root extracts as being related to the heavy metals tolerance of maize and metals accumulation in maize.

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(A) 280 nm

(B) 360 nm

(a)

(b)

(c)

(d)

(e)

p-coumalic

Vanillin

Nariginin

Gallic acid

Myricitin

Epicatechin

(f)

Caffeic acid

Quercetin

Rutin

Weldelolactone

Chlorogenic

Catechin

0 5 10 15 20 25 30 35 40

Retention time (min)

(a)

(b)

(c)

(d)

(e)

Myricitin

(f)

Weldelolactone

Quercetin

Rutin

0 5 10 15 20 25 30 35 40

Retention time (min)

Figure 4.11 HPLC chromatograms of phenolic compounds with retention times of root extracts in 1-week-old maize growing in non-contaminated soil (control) and three levels of Zn/Cd/Pb contaminated soil, as detected at (A) 280 nm and (B) 360 nm. (a) control, (b) low, (c) medium, (d) high, and (e, f) standards of phenolic compounds

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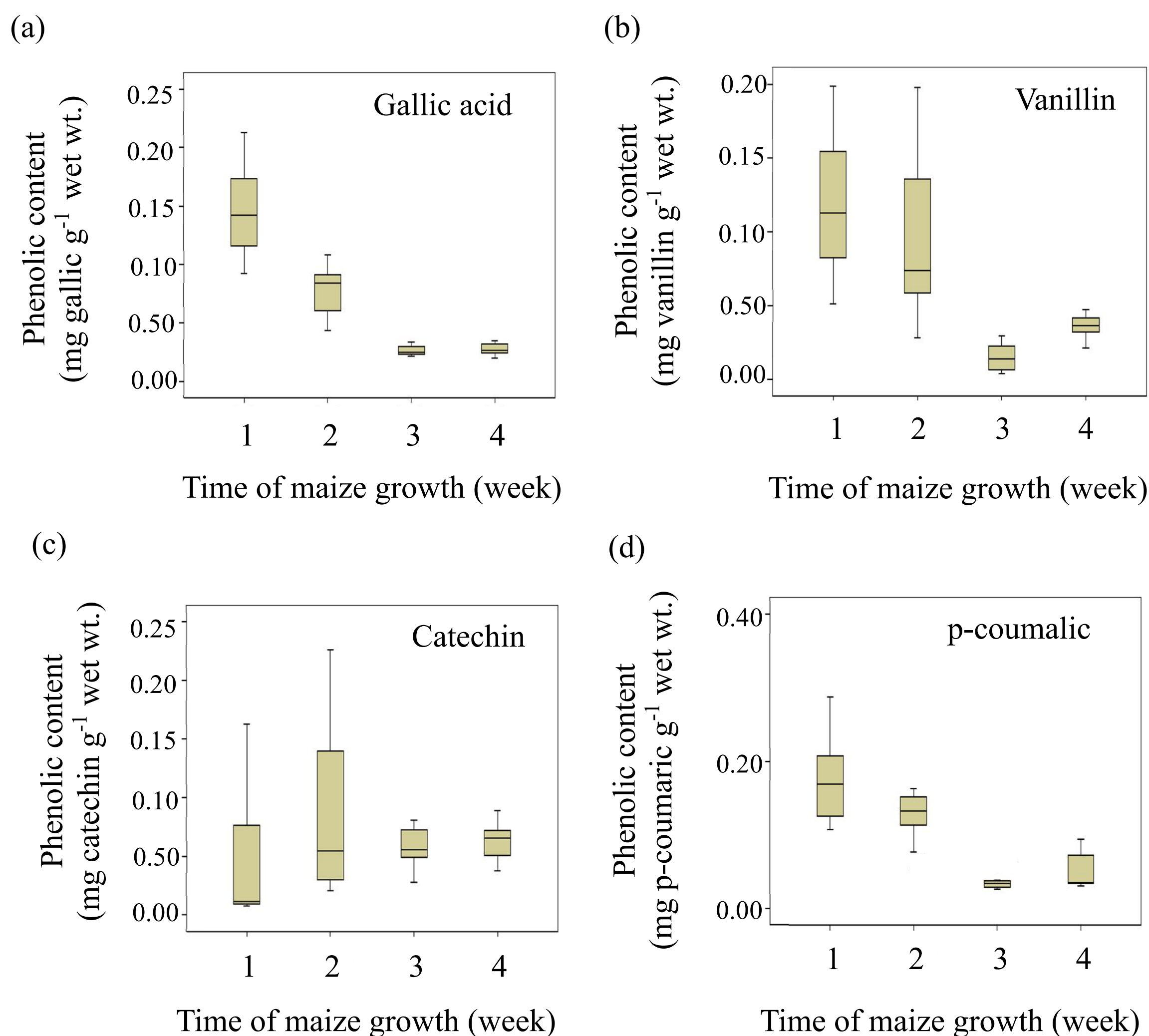

Figure 4.12 Box-plot of concentrations of phenolic compounds in root extracts of maize in weeks 1 to 4. Data set came from sum of phenolic compound from same age of maize, which grew in three levels of Zn/Cd/Pb contaminated soil and non-contaminated soil (control). (a) gallic acid, (b) vanillin, (c) catechin, and (d) p-coumalic (n=12)

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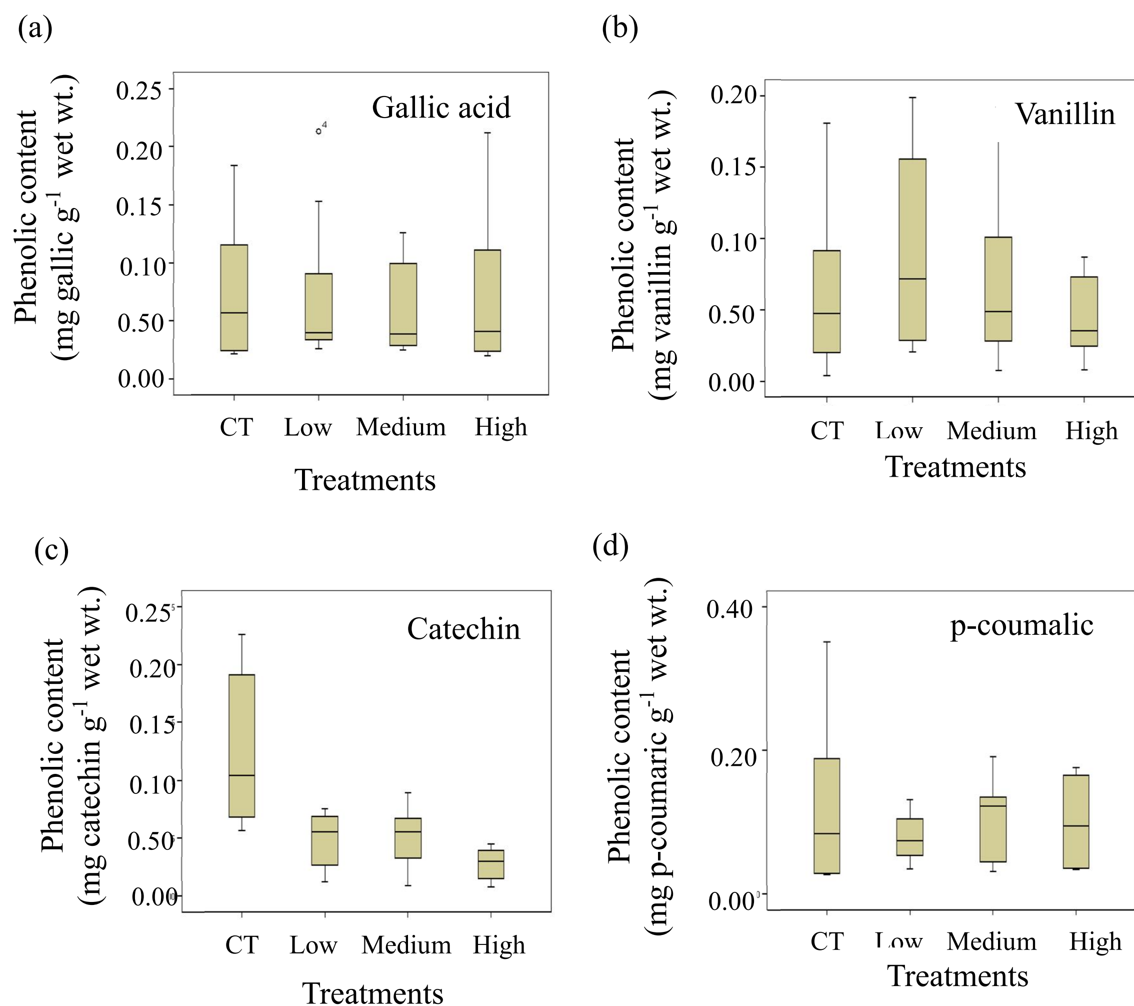

Figure 4.13 Box-plot of concentrations of phenolic compounds in root extracts of maize growing in three levels of Zn/Cd/Pb contaminated soils (low, medium, and high) and non-contaminated soil (control). Data set came from 1 to 4 week-old maize grown in same soil. (a) gallic acid, (b) vanillin, (c) catechin, and (d) p-coumalic (n=12)

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For organic acid, five organic acids (oxalic acid, malic acid, maleic acid, citric acid, and succinic acid) were used as standard samples (Figure 4.14). The HPLC chromatograms of the root extracts obtained from the control and the treatments with four weeks of maize growth were used because this period showed the main peak chromatograms. All the organic acids were found in all the samples. The total concentrations of oxalic acid, malic acid, maleic acid, citric acid, and succinic acid in root extracts of maize grown in non-contaminated soil and three levels of Zn/Cd/Pb contaminated soil are shown in Appendix M. Figure 4.15 shows that oxalic acid, malic acid, maleic acid, and succinic acid had significantly differences in the quantities and trends that decreased during maize growth ($P<0.01$). Zn/Cd/Pb contaminated soil caused a significant decrease in citric acid content in the root extracts when compared with the control ($P<0.01$) (Figure 4.16).

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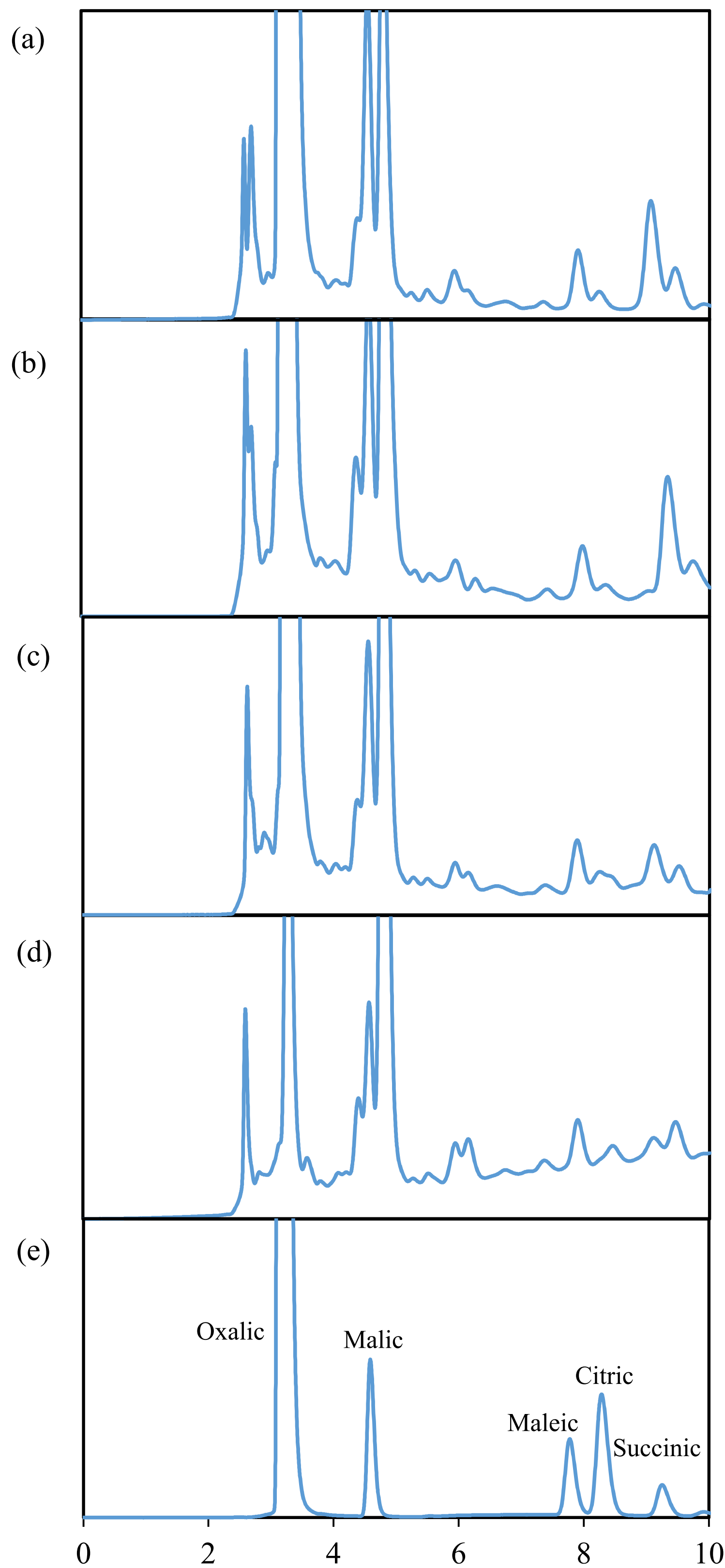

Figure 4.14 HPLC chromatograms of organic acids with retention times in root extracts of four week-old maize growing in non-contaminated soil (control) and three levels of Zn/Cd/Pb contaminated soil. (a) control, (b) low, (c) medium, (d) high, and (e) standards of organic acids

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(a)

Oxalic acid

Organic acid content (mg oxalic g^{-1} wet wt.)

0.30 0.20 0.10 0.00

1 2 3 4

Time of maize growth (week)

(b)

Malic acid

Organic acid content (mg malic g^{-1} wet wt.)

0.25 0.20 0.15 0.10 0.05 0.00

1 2 3 4

Time of maize growth (week)

(c)

Citric acid

Organic acid content (mg citric g^{-1} wet wt.)

0.04 0.02 0.00

1 2 3 4

Time of maize growth (week)

(d)

Succinic acid

Organic acid content (mg succinic g^{-1} wet wt.)

0.04 0.02 0.00

1 2 3 4

Time of maize growth (week)

(e)

Maleic acid

Organic acid content (mg maleic g^{-1} wet wt.)

4.0×10^{-4} 3.0×10^{-4} 2.0×10^{-4} 1.0×10^{-4}

1 2 3 4

Time of maize growth (week)

Figure 4.15 Box-plot of organic acid concentrations in root extracts of maize in weeks 1 to 4. Data set came from sum of phenolic compounds from same age of maize, which grew in three levels of Zn/Cd/Pb contaminated soil and non-contaminated soil (control). (a) oxalic acid, (b) malic acid, (c) citric acid, (d) succinic acid, and (e) maleic acid (n=12)

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Figure 4.16 Box-plot of organic acid concentrations in root extracts of maize growing in three levels of Zn/Cd/Pb contaminated soil (low, medium, and high) and non-contaminated soil (control). Data set came from 1 to 4 week-old maize grown in same soil. (a) oxalic acid, (b) malic acid, (c) citric acid, (d) succinic acid, and (e) maleic acid (n=12)

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The Spearman's rank correlation coefficients in Table 4.12 determined the relationships of the root extract, metals accumulations, maize growth with various Zn/Cd/Pb concentrations, and period of maize growth. The results showed that the increasing period of maize growth caused a decrease in the root exudates, as a negative correlation with the compounds in the root extracts. The phenolic compounds were mainly correlated with maize growth and heavy metals accumulation. This results corresponded to the results of TPC and TFC. Catechin was positively correlated with the Zn/Cd/Pb contaminated soil (*r=-0.664*). On the other hand, the organic acids were mainly correlated with the maize growth, but were less related to the Zn and Cd accumulated in the shoot. Citric acid was negatively correlated with the Zn/Cd/Pb contaminated soil (*r=-0.460*). The soluble Zn from the water extraction was negatively correlated with maleic acid (*r=-0.461*). Only oxalic acid was correlated with Zn and Cd accumulation (*r=0.595* and *r=0.679*). These results indicated that phenolic compounds and flavonoid compounds such as gallic acid, catechin, vanillin, and p_coumalic, which supported heavy metals tolerance in maize and heavy metals accumulation in maize. On the other hand, organic acid, such as oxalic acid, malic acid, maleic acid, citric acid, and succinic acid, which supported heavy metals tolerance in maize. Oxalic acid could support heavy metals accumulation in maize.

The result about the root exudates for phenolic compounds showed that TPC correlated Cd accumulation in the shoot, which was obtained from HPLC results. The phenolic compounds, such as gallic acid, vanillin, and p-coumalic, were correlated with Cd accumulation. However, flavonoid compounds, such as catechin, showed a different negative correlation from TFC with metals contaminated soil and Zn and Cd accumulation in shoots, respectively. This result indicated that catechin maybe related to the mechanism of metal stabilization or metals available in soil. Sgherri *et al.,* (2003) showed that under Cu stress, the main phenolic acids represented in *R. sativus* were chlorogenic, vanillic, caffeic, siringic, p_coumaric, ferulic acids, gallic acid, protocatechuic, and p-hydroxybenzoic acids. Pollock (2010) reported that catechin was a highly reactive compound and redox, it precipitated metals in the form of catechin-metal complexes, catechin-metal-phosphate complexes, and impacted the soil bacterial communities as well as individual bacterial populations. For organic acid, citric acid and maleic acid, was correlated to metals contaminated soil and Zn available, it indicated that

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this organic may be related to the mechanism of metal available or stabilization. In addition, our results showed that phenolic compounds, flavonoid compounds, and organic acids, were important factors for maize to tolerate the heavy metals. The phenolic metabolism in plants as a response to heavy metal stress by possession homeostatic mechanisms that allow them to keep correct concentrations of essential metal ions in cellular compartments and to minimize the damaging effects of an excess of nonessential metals (Michalak, 2006). Carboxylic acids and amino acids, such as citric acids, malic acids, and histidine, are potential ligands for heavy metals and could play a role in the tolerance and detoxification (Hall, 2002). Cd present in the plant extract was in the cationic form by Cd: organic acid interactions were as follows: citric acids >malic acids >aspartic acid (Nigam *et al.,* 2001).

Table 4.12 Spearman's rank correlation coefficient between root extracts, metal accumulations, time of maize growth stages, and treatment with three levels (low, medium, and high) of Zn/Cd/Pb concentrations

Exudates	Treatment	Time	Water-Zn	Shoot-Dry	Acc-Zn	Acc-Cd
Gallic	-0.062	-.853**	-0.253	-.837**	.379**	.551**
Catechin	-.664**	.385**	-.321*	.462**	-.516**	-.705**
Vanillin	-0.099	-.686**	-0.204	-.660**	.373**	.487**
p_coumalic	0.135	-.740**	-0.225	-.735**	.339*	.405**
Oxalic	0.211	-.850**	0.011	-.852**	.595**	.679**
Malic	-0.135	-.601**	-0.253	-.555**	0.193	.287*
Maleic	-0.275	-.659**	-.461**	-.642**	0.042	0.209
Citric	-.460**	-.529**	-.373**	-.443**	-0.089	-0.064
Succinic	0.168	-.287*	-0.243	-.319*	0.137	0.124

4.3.6 Analysis of root exudates by LC-MS/MS

LC-ESI-QTOF-MS/MS was used to identify unknown and unclear compounds in the root extracts. The root extracts obtained from the maize growing in the medium level of Zn/Cd/Pb contaminated soil for four weeks was investigated, because this sample contained all the main peaks in the HPLC chromatogram. The LC-ESI base peak chromatogram (BPC) is presented in Figure 4.17. Table 4.13 presents the mass spectral data obtained in the negative ionization mode for the major compounds and their identification, as well as the characteristic fragmentations and their structure attributions observed in the ESI-MS/MS analysis. The LC-QTOF-MS/MS indicated that a total of 13 individual compounds were detected in the root maize extract, which belong to very different chemicals, including low-molecular weight (phenolic compounds, flavonoid compounds, and organic acids), glycosides, and unidentified compounds. The retention times (RTs) of 4.06 min, 4.34 min, and 13.90 min clearly confirmed the presence of citric (peak 1), malic (peak 2), and p-coumaric acid (peak 12), respectively. Mass spectrum and fragmentation pattern, together with literature data, allowed the identification of other compounds. MS profiling of peaks 3 and 4 were identified as aconitic acid and citraconic acid, respectively (Bylund *et al.,* 2007). Peak 8 was identified as caffeoylquinic acid and it was derivatives, including chlorogenic acid (Shen *et al.,* 2010). Peaks 5, 6, 7, 9, 10, 11, and 13 were identified as possibly furoic acid, dimethyl fumarate, davallioside A, Cyclo-Dopa 5-O-glucoside, DIMBOA glucocide [4-hydroxy-7-methoxy-3-oxo-3,4-dihydro-2H-1,4-benzoxazin-2-yl beta-D-lucopyranoside], 2-Caffeoylisocitrate, and an unidentified compound, respectively. These compounds exhibited a deprotonated molecular ion at m/z 111.0074, 143.0366, 534.1438, 356.0966, 745.1918, 353.0492, and 585.1065, respectively.

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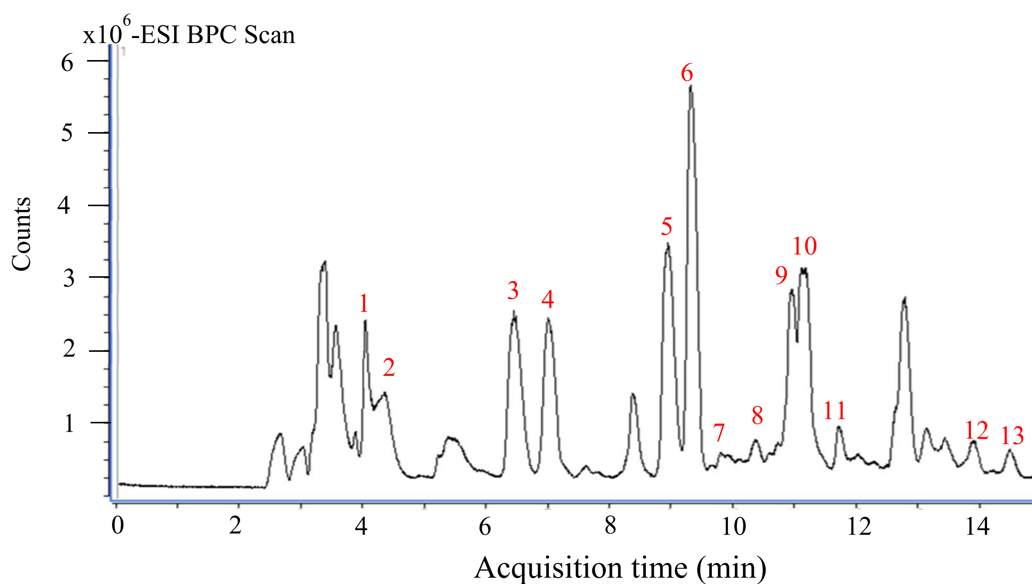

Figure 4.13 LC-ESI base peak chromatogram (BPC) of root extracts from maize growing in medium level of Zn/Cd/Pb contaminated soil obtained from 50% (v/v) methanol fraction. For main peak assignments, see Table 4.13

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Table 4.13 LC-ESI-QTOF-MS/MS analysis of phenolic compounds from root extracts of maize growing in medium level of Zn/Cd/Pb contaminated soil

Number	LC-MS/MS	ESI-MS *m/z* [M-H]	MS/MS-mass fraction									Tentative identification	Formular	Diff (ppm)
1	4.06	191.0181	191.018	154.996	133.011	111.008	72.993					Citric acid	$C_6H_8O_7$	8.47
2	4.37	133.0131	133.0131	115.003	71.0132							Malic acid	$C_4H_6O_5$	8.56
3	5.40	173.0077	173.0077	129.0181	85.0287							Aconitic Acid	$C_6H_6O_6$	8.4
4	7.06	129.0181	129.0181	98.9035	85.0289	55.4652						Citraconic acid	$C_5H_6O_4$	9.48
5	8.91	111.0074	111.0074	68.9951	67.0183							Furoic acid	$C_5H_4O_3$	12.21
6	9.29	143.0336	143.0336	111.0075	67.0183							Dimethyl maleate	$C_6H_8O_4$	9.6
7	9.84	534.1438	470.0888	402.102	372.0908	192.0276	164.0333	149.0098	89.0203	59.0133		Davallioside A	$C_{25}H_{29}O_{12}$	33.51
8	10.41	353.0852	353.0852	191.054	135.0432	112.9843	85.0285					caffeoyl quinic acid (Chlorogenic)	$C_{16}H_{18}O_9$	7.36
9	10.95	356.0966	356.0966	194.044	138.0544	59.0133						cyclo-Dopa 5-O-glucoside	$C_{15}H_{19}O_9$	5.54
10	11.20	745.1918*	372.0914	210.0388	187.023	164.0335	143.0335	111.0075				DIMBOA glucocide	$C_{15}H_{19}NO_{10}$	5.94
11	11.74	353.0492	353.0492	173.0074	112.9843	111.0076	85.0286					2-Caffeoylisocitrate	$C_{15}H_{14}O_{10}$	6.27
12	13.90	163.0387	163.0387	121.0281	119.0491	93.0337	65.039					p-Coumaric acid	$C_9H_8O_3$	8.34
13	14.52	585.1065	585.1065	379.0642	295.0436	257.0284	213.0376	181.0122	154.0255	112.9845	99.0073	Unidentified	$C_{28}H_{26}O_4$	31.58

*[2M-H]

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For the pot experiment, the heavy metals contaminated soil did not affect maize growth and chlorophyll contents, when compared with the maize growing in the control soil. The results supported the results of the filed study, and they implied that the maize had the ability to tolerate the Zn, Cd, and Pb contaminated soil. Growing maize in contaminated soil increased the metals available. A key element in the acquisition of nutritional metals is the release of exudates with chelator properties from the roots into the rhizosphere, which can select growth substrates for soil microorganisms. One related process concerns the role of root exudates in metal tolerance by metal chelators (Hall, 2002). On the one hand, it can be used as a defense strategy by producing less soluble metal complexes unsuitable for entering the plant (Viehweger, 2014). The result about root exudates for phenolic compound showed that TPC correlated with Cd accumulation in the shoot, which was obtained from the HPLC result. The phenolic compounds of gallic acid, vanillin, and p-coumalic were moderately positively correlated with Cd accumulation. However, flavonoid compounds, such as catechin, showed different correlations from TFC as strongly negative with metals contaminated soil and moderate and strong negatively with Zn and Cd accumulation in the shoot, respectively. This result indicates that catechin maybe related to the mechanism of metal stabilization or metals available in soil. However, Pollock (2010) reported that catechin was a highly reactive compound and redox, it precipitated metals in the form of catechin-metal complexes, catechin-metal-phosphate complexes, and impacted soil bacterial communities as well as individual bacterial populations. For organic acids, citric acid and maleic acid were moderately negatively correlated metals contaminated soil and Zn availability, it indicated that this organic maybe related to the mechanism of metal availability or stabilization. In addition, our results showed that phenolic compounds, flavonoid compounds, and organic acids were important factors for maize to tolerate heavy metals. Phenolic metabolism in plants is a response to heavy metal stress by possession of homeostatic mechanisms that allow them to keep the correct concentrations of essential metal ions in cellular compartments and to minimize the damaging effects of an excess of nonessential metals (Michalak, 2006). Carboxylic acids and amino acids, such as citric acids, malic acids, and histidine, are potential ligands for heavy metals and could play a role in tolerance and detoxification (Hall, 2002). The Cd present in the plant extract was

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in the cationic form by Cd, and the organic acid interactions were as follows: citric acids >malic acids >aspartic acid (Nigam *et al.,* 2001).

The result of LC-MS/MS showed many compounds in the root extracts. All of the samples in the HPLC were found in the subunit of compounds in the result of LC-MS/MS. This method showed high reproducibility to determine simultaneously 13 individual compounds in the root extracts. Generally, aconitic acid is an intermediate in the isomerization of citrate to isocitrate in the citric acid cycle (Aconitic acid, Wikipedia: The Free Encyclopedia). It acted upon the enzyme aconitase. Whereas, citraconic is the *cis*-isomer of mesaconic acid, which was obtained from citric acid (Citraconic acid, Wikipedia: The Free Encyclopedia). The hydrolysis of dimethyl maleate gives maleic acid, or possibly the maleic acid monomethyl ester. Hydration of the same compound gives malic acid (Dimethyl maleate, Wikipedia: The Free Encyclopedia). Cheng-Bin *et al.,* (1990) reported that davallioside A was a diastereoisomer of the epicatechin compound. Epicatechin and catechin, as well as their gallic acid conjugates, were ubiquitous constituents of vascular plants. Caffeoylquinic acid was an ester of caffeic acid and (-)-quinic acid (Mongkhonsin *et al.,* 2016). Erb *et al.,* (2009) showed roots containing higher amounts of caffeic acid, p_coumaric acid, ferulic acid, and sinapic acid during the early maize growth within 10 to 12 days. Takahama *et al.,* (1999) showed that the chlorogenic (caffeoylquinic acid) content was correlated with the peroxidase activity in the apoplast. DIMBOA was a secondary metabolite that occurs in high abundance as glucosides in the Poaceae, among them the cereals maize (*Zea mays*), wheat (*Triticum aestivum*), and rye (*Secale cereale*). (Nikus, 2003). Maksimovic *et al.,* (2008) reported that phenolic compounds, such as p-coumaric, in the maize root fluid exhibited the presence of lignin precursors (coniferyl alcohol and p-coumaric acid), which can regulate growth in different ways, being associated with cell elongation processes. Lignification can serve as a barrier that limits the entry of metals into the tissue (Mongkhonsin *et al.,* 2016). In addition, Li *et al.,* (2016) reported that the root exudates of maize included signal molecules that likely played an important role in activating rhizobia. The change quantity of the root exudates, especially organic acid, may increase the effectiveness of phytoremediation under flood, drought, and nutrient stress (Henry *et al.*, 2007). Therefore, the root exudates of maize had a beneficial interaction between plant, soil, and microbes.

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CHAPTER 5

CONCLUSIONS AND SUGGESTIONS

5.1 Conclusions

Chemical characterization of agricultural soil near Mae-tow Creek in Mae Sot, Tak Province, Thailand revealed high concentrations of Zn, Cd, and Pb. Concentrations of heavy metals in our field site gradually decreased from the source of irrigation as 302-3,132 mg Zn kg^{-1}, 5-66 mg Cd kg^{-1}, 30-136 mg Pb kg^{-1}, and 9,070-18,108 mg Fe kg^{-1}, which were higher than the maximum allowable concentrations (MAC) in agricultural soils. Rainfall and irrigation through drains and flooding were the main factors of the bioavailability of heavy metals in the field. The lower Shannon-Wiener index of the cultureable bacterial community in the rhizospheric soil than in the bulk soil was caused by the effect of plant species and plant metabolic stress, which could select a specific rhizobacteria. The change in culturable rhizobacterial communities was related to growth stages, rainfall, and temperature. The culturable rhizobacteria found in every stage of maize growing in the heavy metals contaminated soil were *Brevibacillus agri* (KY618802), *Bacillus* sp. (KY629623 and KY629626), *Cellulosimicrobium funkei* (KY629624), and *Pseudomonas chlororaphis* (KY629627). PCR-DGGE clearly showed that the heavy metals contaminated soil caused a shift in the total bacterial community in the rhizosphere. Although abiotic factors in the agriculture soil did not have a significant correlation with the metals accumulation in the maize, the culturable rhizobacteria and the maize growth stages significantly affected the Zn and Cd accumulated in the phytomass ($P<0.01$). Zn and Cd were mainly accumulated in the root part, and there was a high Zn concentration in the edible maize seeds with a limit of Cd for applying as animal feed. The gradually reducing values of the *pollution index* (C_f) and *potential ecological risk index* (E_f) indicated that growing maize in this area could remediate the soil.

Under the pot experiment, levels of the Zn/Cd/Pb contaminated soil were positively correlated with the Zn available in water and Zn and Cd accumulation in the shoot. Root extracts indicated that phenolic compounds and flavonoid compounds were the main factors for maize growth and metals accumulation (Zn and Cd), whereas most

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of organic acids related to maize growth. Only the catechin part of the TFC showed a negative correlation with the metals contamination and Zn and Cd accumulation in the shoot. Citric acid was negatively correlated to the metals contaminated soils, whereas maleic acid and Zn available in the water extracts had a moderate negative correlation. Oxalic acid was only correlated with Zn and Cd accumulation. The LC-MS/MS showed high reproducibility to determine simultaneously 13 individual compounds in the root extracts, which were phenolic compounds, flavonoid compounds, and organic acids, such as p_coumaric, caffeoylquinic acid, chlorogenic, citric, malic, aconitic, and citraconic as well as some glycoside compounds.

5.2 Suggestions

5.2.1 The results from this field study indicated that maize has potential applications in a strategy of soil remediation. Therefore, using maize phytomanagement to decontaminate soil couple with management of irrigation from Mae Tao Creek should be considered to be applied in the field site for farm enterprise and environment sustainability.

5.2.2 The culturable rhizobacteria isolated from the metals contaminated soils of the maize field may have properties of plant growth promotion, and they should be studied further, especially their application in phytoremediation in the area.

5.2.3 The PCR-DGGE technique is a good method to study uncultureable and cultureable microbial communities. Therefore, PCR preparation should be studied further to obtain longer fragments of more than 200 bp for the propose of rhizobacterial identification.

5.2.4 The results obtained from the pot study indicated that the root extracts may be related with many cellular mechanisms of metal tolerance and/or metal accumulation in maize. Therefore, the root extracts should be studied further.

5.2.5 This research showed a limit to the study of root exudates in the field experiment. Therefore, a field experimental design to collect and study root exudates should be considered and studied further.

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APPENDICES

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Appendix A

Field experiment

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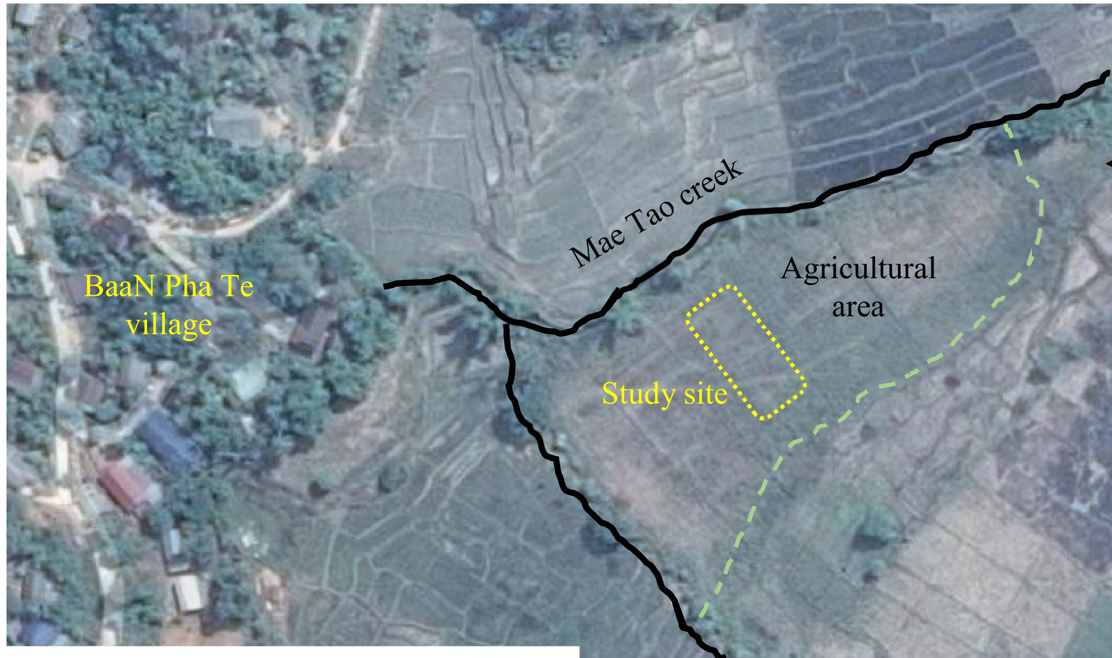

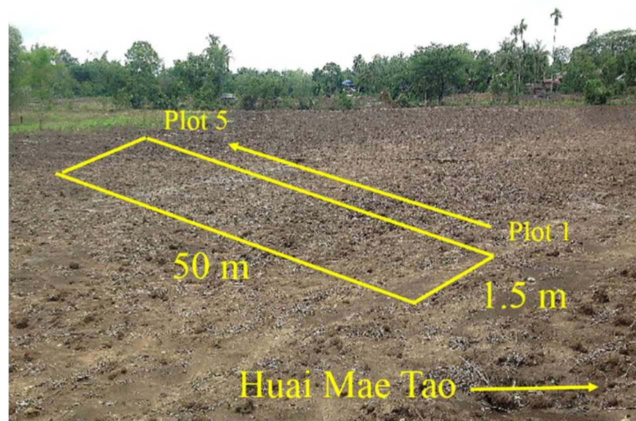

Appendix A-1 Study area in Ban Pha Te, Phatat Phadaeng Sub-district, Mae Sot District, Tak Province, Thailand and concentrations of heavy metals

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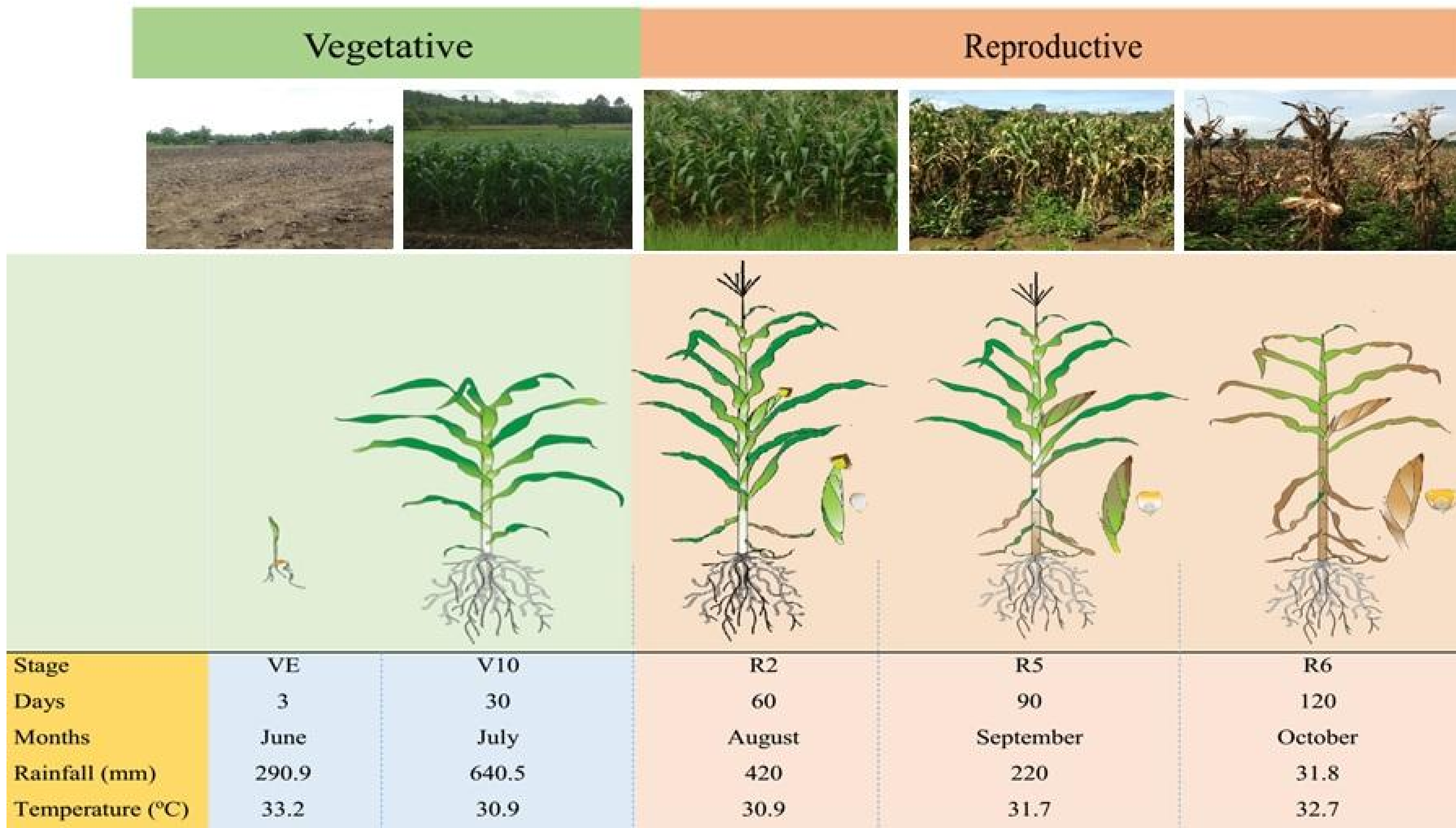

Stage	VE	V10	R2	R5	R6
Days	3	30	60	90	120
Months	June	July	August	September	October
Rainfall (mm)	290.9	640.5	420	220	31.8
Temperature (°C)	33.2	30.9	30.9	31.7	32.7

Appendix A-2 Stages of maize development, rainfall and temperature (Modified picture from Ciampitti *et al.*, 2016)

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Appendix A-3**.** Chemical properties of bulk and rhizospheric soils and Shannon-Wiener Index (n=3)

Variable	Rhizosphere (n=60)			Bulk soil (n=60)		
	Min	Max	Mean	Min	Max	Mean
pH	5.86	7.70	6.68	5.94	7.90	6.78
EC (µs cm^{-1})	38.00	205.00	101.25	32.00	224.00	101.58
Om (%)	2.94	5.93	4.34	2.91	7.02	4.35
P (mg kg^{-1})	20.33	46.88	30.55	15.85	38.75	26.56
K (mg kg^{-1})	90.71	211.53	162.57	78.84	200.76	124.11
N (%)	0.17	0.25	0.21	0.17	0.33	0.21
CEC (cmol kg^{-1})	9.81	13.79	11.09	8.54	17.08	11.62
Moisture (%)	9.44	25.37	16.93	1.54	24.72	13.66
DTPA-Zn (mg kg^{-1})	104.71	223.41	168.40	45.38	214.87	144.45
DTPA-Cd (mg kg^{-1})	4.89	24.14	12.90	3.36	24.03	10.38
DTPA-Pb (mg kg^{-1})	9.71	38.87	21.82	8.30	40.51	20.32
DTPA-Fe (mg kg^{-1})	31.25	405.77	125.12	29.36	232.76	99.39
Total-Zn (mg kg^{-1})	526.47	4,732.09	1,528.91	222.46	3,917.76	1,543.59
Total-Cd (mg kg^{-1})	6.68	67.15	23.16	4.97	69.31	23.44
Total-Pb (mg kg^{-1})	26.12	132.90	63.71	5.33	141.20	63.99
Total-Fe (mg kg^{-1})	7,953.18	18,820.86	12,636.68	1,624.00	20,219.49	12,731.84
Bacteria count (Log CFU/g soil dry wt)	7.39	8.73	8.09	6.59	7.55	7.01
Shannon-Wiener index	0.41	2.28	1.46	1.01	3.07	1.98

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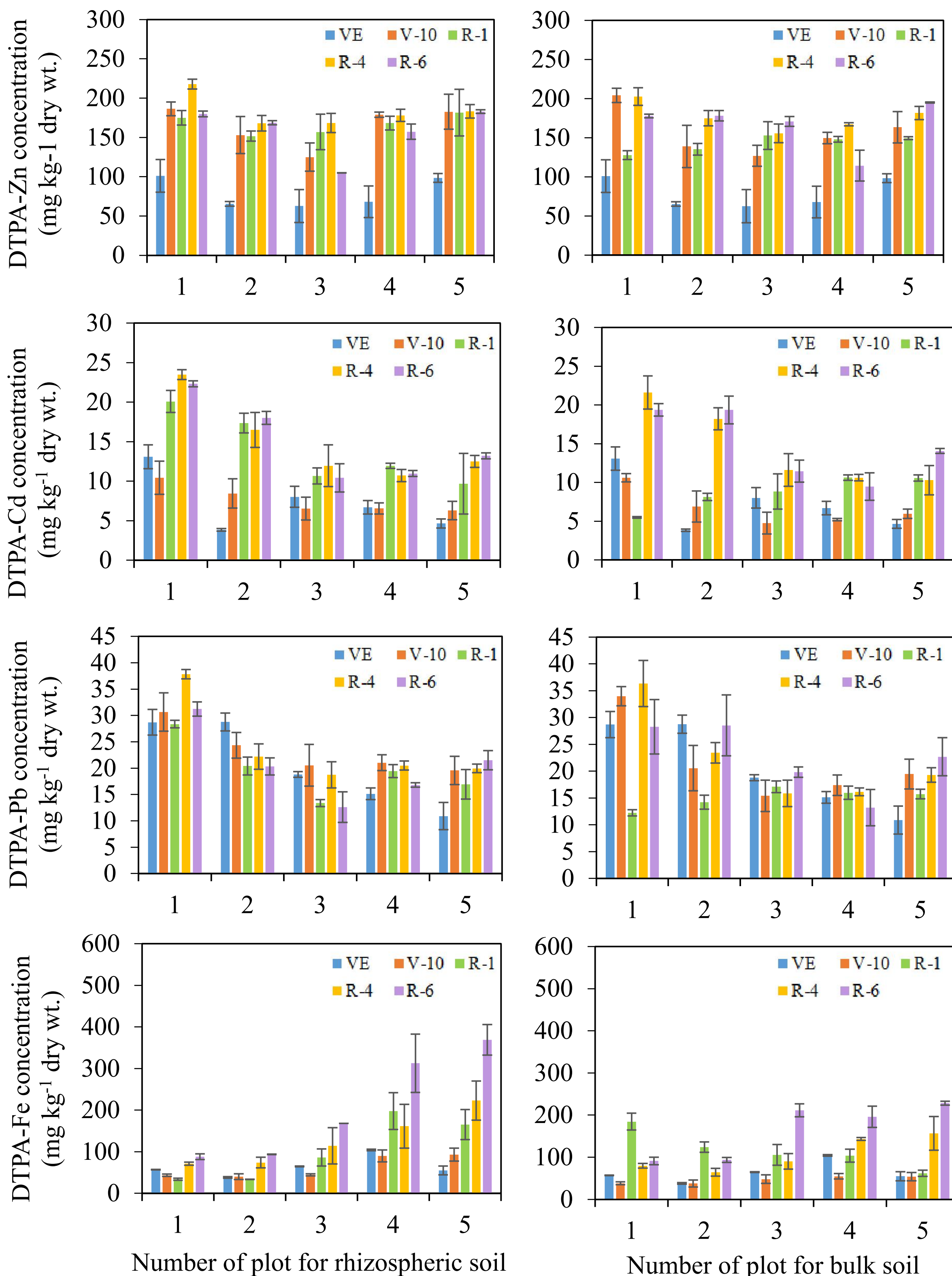
Appendix A-4 Total concentrations of heavy metals in rhizospheric and bulk soils at various distances and stages of maize development (n=3)



Appendix A-5 DTPA concentrations of heavy metals in rhizospheric and bulk soils at various distance and stages of maize development (n=3)

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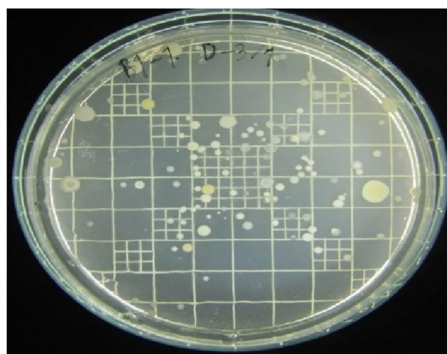
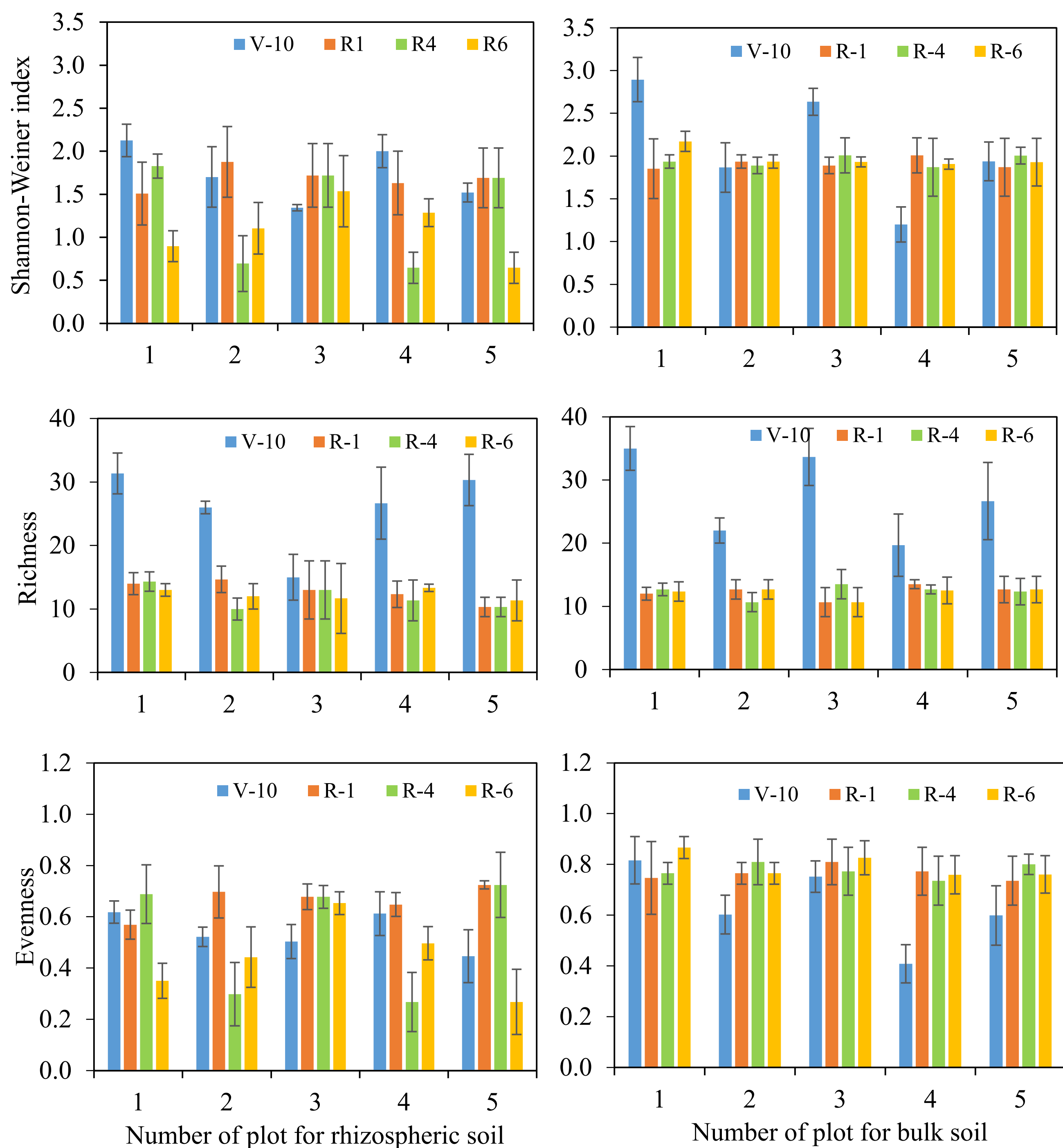


Number	pinpiont	Circular	Filamentous	Rhiziod	Irregular	Entire	Undulate	Lobate	Erose	Filamentous	Curled	Flat	Raised	Convex	Pulvinate	Umbonate	Dry	Smooth	Rought	Mucoid	Wrinkled	Glistenning	Opaque	Translucent	Opalecent	Transparent	Photogenic	Colour
3		■					■						■								■		■					ใสอมเขียว
2		■				■							■						■				■					ครีม
2		■					■			■						■		■						■				ครีมอมเหลือง
2		■				■								■					■							■		ใสอมเขียว
2		■					■									■		■						■				ครีม
2		■						■								■				■				■				ครีม
12		■					■						■								■		■					ครีม
7		■				■							■						■				■					ขาวใส
28		■					■									■		■						■				ครีมอมเหลือง
9		■				■								■					■							■		ครีม
2		■					■									■		■						■				ครีม
2		■						■								■				■				■				ครีม
2		■					■									■		■						■				ครีมอมเหลือง
40	■																											ครีม

Group	Number Specific group (Ni)	Total Number (N)	Ni/N	ln Ni/N	(Ni/N)Ln(Ni/N)	Shannon-Wiener Index (H)	Richness (S)	Evenness (E_H)
1	3	115	0.026	-3.646	-0.095	1.976	14	0.749
2	2		0.017	-4.052	-0.070			
3	2		0.017	-4.052	-0.070			
4	2		0.017	-4.052	-0.070			
5	2		0.017	-4.052	-0.070			
6	2		0.017	-4.052	-0.070			
7	12		0.104	-2.260	-0.236			
8	7		0.061	-2.799	-0.170			
9	28		0.243	-1.413	-0.344			
10	9		0.078	-2.548	-0.199			
11	2		0.017	-4.052	-0.070			
12	2		0.017	-4.052	-0.070			
13	2		0.017	-4.052	-0.070			
14	40		0.348	-1.056	-0.367			

Appendix A-6 Determination of bacterial counts and culturable bacterial community



Appendix A-7 Shannon-Wiener indices (*H*), richness (*S*), and evenness in rhizospheric and bulk soils at various distance and stages of maize development (n=3)

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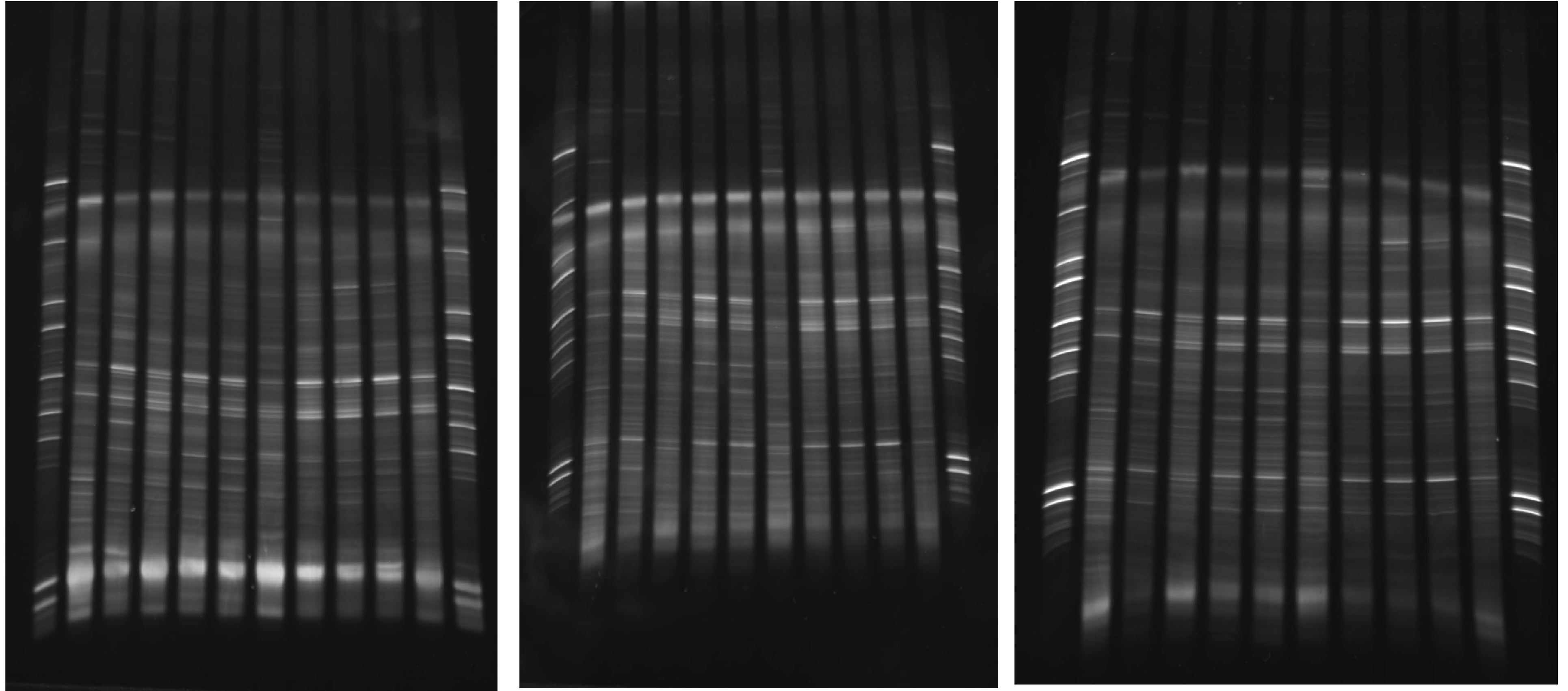

Appendix A-8 PCR-DGGE

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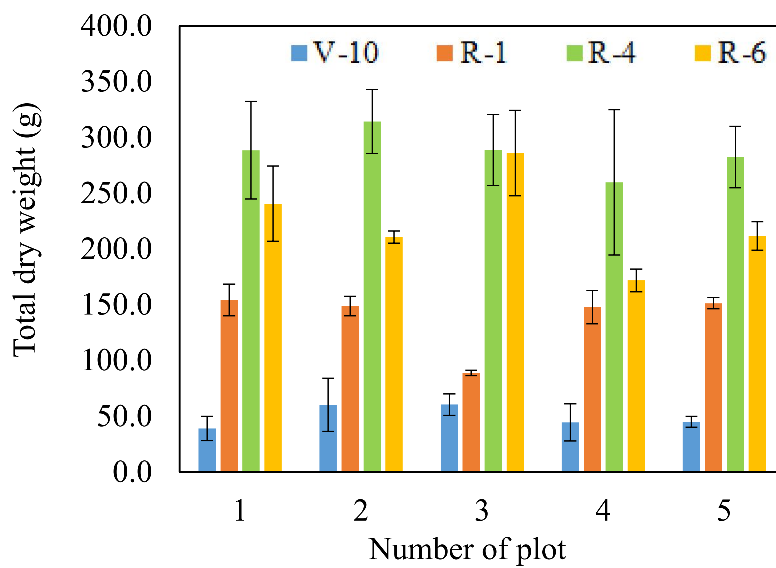

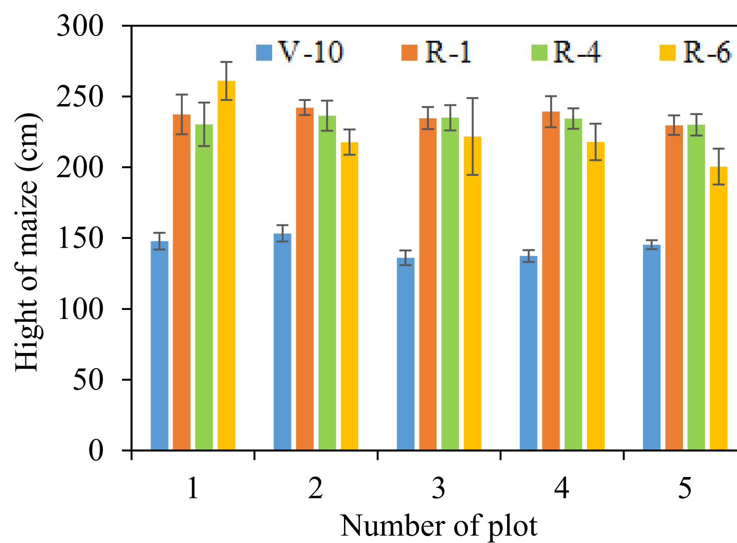

Appendix A-9 Total dry weight and height of maize (n=3)

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Appendix A-10 Accumulation of Zn and Cd in roots and shoots of maize (n=3)

Metals	Plot	Metals accumulation in root (mg kg^{-1})				Metals accumulation in shoot (mg kg^{-1})			
		30 day	60 day	90 day	120 day	30 day	60 day	90 day	120 day
Zn	1	453.79±95.40	268.41±73.87	218.04±11.56	126.80±13.96	469.22±18.12	235.71±31.26	244.48±50.96	259.59±9.52
	2	166.26±25.22	149.56±46.86	198.94±63.92	255.23±41.23	432.73±45.95	186.75±22.95	227.01±24.03	348.75±11.87[c]
	3	247.30±45.77	142.80±20.64	220.14±25.13	257.37±30.49	446.37±128.98	294.45±12.49	286.12±6.55	218.52±44.71
	4	352.20±99.82	151.73±27.56	182.55±13.64	403.43±54.38	537.14±44.19	269.62±19.29	359.80±9.46	233.47±25.46
	5	311.83±58.21	153.51±48.47	240.28±28.71	280.49±30.07	485.27±44.09	264.68±17.84	240.66±40.54	394.31±83.90
Cd	1	23.05±4.29	19.09±8.41	11.41±3.23	11.84±0.22	10.66±1.21	nd	nd	nd
	2	16.42±3.82	12.47±1.52	12.08±4.87	12.71±1.43	11.29±1.48	nd	nd	nd
	3	19.03±5.25	8.35±0.44	13.30±4.46	17.66±1.18	12.73±1.89	nd	nd	nd
	4	18.92±4.09	10.28±0.95	11.35±0.92	20.98±0.77	12.72±1.46	nd	nd	nd
	5	17.35±1.34	9.38±0.28	13.03±2.60	27.33±0.93	12.64±1.04	nd	nd	nd

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Appendix A-11 Translocation factor (TF) and biological absorption coefficient (BAC) (n=3)

Translocation factor (TF) of Zn in maize

Plot	Maize growth (days)			
	30	60	90	120
1	1.06±0.20	0.90±0.14	1.13±0.28	2.06±0.24
2	2.62±0.27	1.34±0.47	1.25±0.54	1.39±0.25
3	1.79±0.36	2.08±0.23	1.31±0.19	0.85±0.14
4	1.60±0.41	1.81±0.31	1.98±0.16	0.59±0.09
5	1.59±0.33	1.81±0.39	1.01±0.19	1.42±0.39

Biological Absorption Coefficient (BAC) Zn of root maize

Plot	Maize growth (days)			
	30	60	90	120
1	0.16±0.04	0.07±0.01	0.08±0.00	0.06±0.01
2	0.07±0.01	0.07±0.01	0.14±0.03	0.22±0.03
3	0.22±0.04	0.14±0.03	0.23±0.02	0.35±0.06
4	0.26±0.05	0.15±0.03	0.21±0.04	0.65±0.11
5	0.31±0.04	0.14±0.03	0.29±0.02	0.27±0.05

Biological Absorption Coefficient (BAC) Cd of root maize

Plot	Maize growth (days)			
	30	60	90	120
1	0.42±0.07	0.32±0.09	0.20±0.05	0.32±0.02
2	0.61±0.06	0.33±0.04	0.42±0.10	0.81±0.21
3	1.70±0.52	0.56±0.05	1.08±0.24	2.08±0.64
4	1.63±0.43	0.75±0.05	1.07±0.09	1.75±0.19
5	1.48±0.21	0.76±0.20	1.08±0.18	1.79±0.19

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Appendix B

Pot experiment

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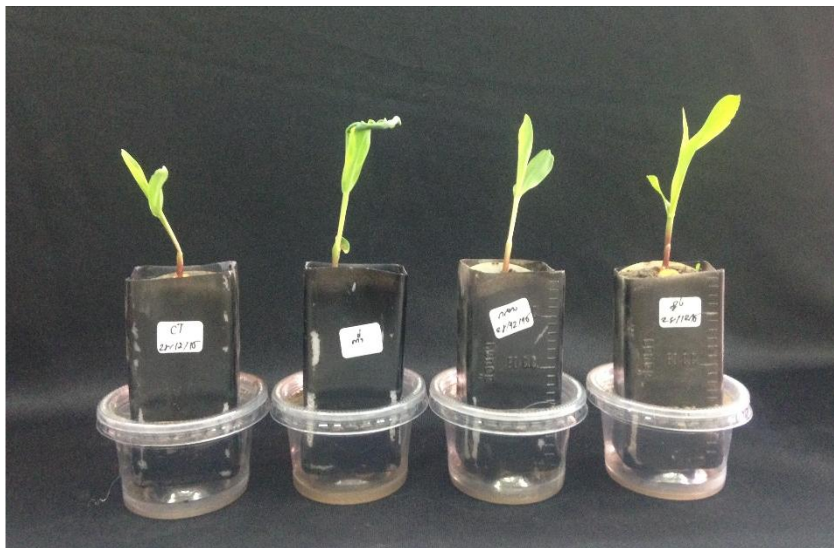

Appendix B-1 Pot design

Appendix B-2 Content of phenolic compounds in root extracts (n=3)

Root extract	Treatments	Duration for study			
		1 weeks	2 weeks	3 weeks	4 weeks
Gallic acid					
	CT	0.157±0.026	0.093±0.006	0.023±0.001	0.026±0.001
	Low	0.153±0.060	0.067±0.022	0.034±0.000	0.030±0.004
	Medium	0.118±0.008	0.067±0.023	0.026±0.001	0.033±0.001
	High	0.163±0.049	0.081±0.027	0.025±0.002	0.022±0.002
Catechin					
	CT	0.145±0.017	0.223±0.003	0.069±0.012	0.068±0.003
	Low	0.019±0.007	0.041±0.013	0.074±0.002	0.061±0.005
	Medium	0.010±0.001	0.058±0.003	0.055±0.000	0.081±0.008
	High	0.009±0.001	0.026±0.006	0.036±0.008	0.041±0.004
Vanillin					
	CT	0.145±0.036	0.067±0.008	0.005±0.001	0.035±0.001
	Low	0.164±0.035	0.147±0.050	0.025±0.004	0.037±0.010
	Medium	0.084±0.033	0.125±0.067	0.012±0.004	0.043±0.003
	High	0.080±0.007	0.050±0.022	0.018±0.010	0.031±0.009
p_cumalic					
	CT	0.288±0.064	0.143±0.010	0.027±0.000	0.033±0.002
	Low	0.120±0.012	0.077±0.023	0.053±0.018	0.060±0.025
	Medium	0.154±0.037	0.129±0.003	0.034±0.003	0.095±0.043
	High	0.171±0.005	0.157±0.006	0.036±0.003	0.036±0.000

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Appendix B-3 Content of organic acids in root extracts (n=3)

Root extract	Treatments	Duration for study			
		1 weeks	2 weeks	3 weeks	4 weeks
Oxalic acid (mg g^{-1} wet wt)					
	CT	1.955±0.243	0.477±0.160	0.034±0.004	0.048±0.005
	Low	2.594±0.213	0.835±0.048	0.532±0.137	0.400±0.117
	Medium	2.415±0.172	0.780±0.025	0.342±0.097	0.300±0.015
	High	2.284±0.143	1.286±0.196	0.383±0.003	0.374±0.021
Malic acid (mg g^{-1} wet wt)					
	CT	0.205±0.011	0.100±0.016	0.024±0.005	0.057±0.009
	Low	0.113±0.014	0.079±0.014	0.063±0.010	0.073±0.006
	Medium	0.137±0.021	0.072±0.001	0.049±0.006	0.072±0.006
	High	0.071±0.004	0.071±0.014	0.048±0.011	0.069±0.002
Maleic acid (µg g^{-1} wet wt)					
	CT	0.318±0.038	0.275±0.050	0.070±0.023	0.142±0.015
	Low	0.240±0.020	0.116±0.012	0.104±0.002	0.112±0.025
	Medium	0.253±0.026	0.119±0.003	0.085±0.015	0.091±0.017
	High	0.135±0.007	0.133±0.015	0.091±0.015	0.101±0.006
Citric acid (µg g^{-1} wet wt)					
	CT	12.40±2.06	34.22±0.62	15.87±2.49	4.98±1.45
	Low	5.09±1.39	6.47±2.74	7.34±0.95	3.90±1.62
	Medium	10.94±3.04	6.41±0.92	5.52±1.20	2.78±0.93
	High	6.12±0.51	6.25±0.53	3.80±1.78	3.73±0.44
Succinic acid (µg g^{-1} wet wt)					
	CT	32.02±2.87	32.57±0.13	5.18±0.38	19.15±1.99
	Low	33.52±3.20	26.51±0.85	14.08±2.43	16.55±2.92
	Medium	36.94±5.64	28.21±3.16	12.51±0.97	32.28±3.86
	High	20.22±2.43	33.36±1.31	10.60±1.30	43.78±3.60

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Appendix C

Data for SPSS analysis

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Appendix C-1 Normalization and randomness of chemical properties of bulk and rhizospheric soils and bacterial community

1. pH

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
pH-plot1	3	7.4867	.16166	7.34	7.66
pH-plot2	3	6.3300	.11136	6.21	6.43
pH-plot3	3	6.4867	.07767	6.40	6.55
pH-plot4	3	6.5167	.09018	6.43	6.61
pH-plot5	3	6.4567	.09504	6.36	6.55

One-Sample Kolmogorov-Smirnov Test

		pH-plot1	pH-plot2	pH-plot3	pH-plot4	pH-plot5
N		3	3	3	3	3
Normal Parameters(a,b)	Mean	7.4867	6.3300	6.4867	6.5167	6.4567
	Std. Deviation	.16166	.11136	.07767	.09018	.09504
Most Extreme Differences	Absolute	.232	.238	.285	.196	.181
	Positive	.232	.193	.207	.196	.179
	Negative	-.192	-.238	-.285	-.183	-.181
Kolmogorov-Smirnov Z		.402	.412	.493	.340	.313
Asymp. Sig. (2-tailed)		.997	.996	.968	1.000	1.000

a Test distribution is Normal.

b Calculated from data.

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Runs Test

	pH-plot1	pH-plot2	pH-plot3	pH-plot4	pH-plot5
Test Value(a)	7.46	6.35	6.51	6.51	6.46
Cases < Test Value	1	1	1	1	1
Cases >= Test Value	2	2	2	2	2
Total Cases	3	3	3	3	3
Number of Runs	3	3	2	3	2
Z	.354	.354	.000	.354	.000
Asymp. Sig. (2-tailed)	.724	.724	1.000	.724	1.000

a Median

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2. EC

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Ec-plot1	3	191.6667	18.92969	170.00	205.00
Ec-plot2	3	165.5000	12.37942	151.50	175.00
Ec-plot3	3	95.3000	4.28836	91.60	100.00
Ec-plot4	3	79.1667	8.83648	69.00	85.00
Ec-plot5	3	72.5000	7.30821	65.40	80.00

One-Sample Kolmogorov-Smirnov Test

		Ec-plot1	Ec-plot2	Ec-plot3	Ec-plot4	Ec-plot5
N		3	3	3	3	3
Normal Parameters(a,b)	Mean	191.66	165.500	95.300	79.166	72.500
	Std. Deviation	18.929	12.379	4.288	8.836	7.308
Most Extreme Differences	Absolute	.337	.309	.259	.355	.188
	Positive	.241	.221	.259	.255	.188
	Negative	-.337	-.309	-.197	-.355	-.181
Kolmogorov-Smirnov Z		.583	.534	.448	.614	.326
Asymp. Sig. (2-tailed)		.886	.938	.988	.845	1.000

a Test distribution is Normal.

b Calculated from data.

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Runs Test

	Ec-plot1	Ec-plot2	Ec-plot3	Ec-plot4	Ec-plot5
Test Value(a)	200.00	170.00	94.30	83.50	72.10
Cases < Test Value	1	1	1	1	1
Cases >= Test Value	2	2	2	2	2
Total Cases	3	3	3	3	3
Number of Runs	2	3	3	3	2
Z	.000	.354	.354	.354	.000
Asymp. Sig. (2-tailed)	1.000	.724	.724	.724	1.000

a Median

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3. OM

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Om-plot1	3	6.3900	.90736	5.35	7.02
Om-plot2	3	5.5967	.51598	5.10	6.13
Om-plot3	3	4.3900	.48570	3.92	4.89
Om-plot4	3	3.6433	.32347	3.27	3.84
Om-plot5	3	3.4800	.43347	2.98	3.75

One-Sample Kolmogorov-Smirnov Test

		Om-plot1	Om-plot2	Om-plot3	Om-plot4	Om-plot5
N		3	3	3	3	3
Normal Parameters(a,b)	Mean	6.3900	5.5967	4.3900	3.6433	3.4800
	Std. Deviation	.90736	.51598	.48570	.32347	.43347
Most Extreme Differences	Absolute	.341	.195	.191	.374	.369
	Positive	.244	.195	.191	.272	.267
	Negative	-.341	-.183	-.182	-.374	-.369
Kolmogorov-Smirnov Z		.591	.338	.331	.648	.639
Asymp. Sig. (2-tailed)		.876	1.000	1.000	.795	.809

a Test distribution is Normal.

b Calculated from data.

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Runs Test

	Om-plot1	Om-plot2	Om-plot3	Om-plot4	Om-plot5
Test Value(a)	6.80	5.56	4.36	3.82	3.71
Cases < Test Value	1	1	1	1	1
Cases >= Test Value	2	2	2	2	2
Total Cases	3	3	3	3	3
Number of Runs	2	2	2	2	2
Z	.000	.000	.000	.000	.000
Asymp. Sig. (2-tailed)	1.000	1.000	1.000	1.000	1.000

a Median

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4. P

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
P-plot1	3	20.1733	.34196	19.78	20.40
P-plot2	3	24.8933	.18502	24.68	25.01
P-plot3	3	17.7067	.31533	17.43	18.05
P-plot4	3	30.2133	.22502	30.05	30.47
P-plot5	3	26.5300	.18520	26.32	26.67

One-Sample Kolmogorov-Smirnov Test

		P-plot1	P-plot2	P-plot3	P-plot4	P-plot5
N		3	3	3	3	3
Normal Parameters(a,b)	Mean	20.1733	24.8933	17.7067	30.2133	26.5300
	Std. Deviation	.34196	.18502	.31533	.22502	.18520
Most Extreme Differences	Absolute	.354	.366	.250	.328	.314
	Positive	.254	.264	.250	.328	.225
	Negative	-.354	-.366	-.195	-.234	-.314
Kolmogorov-Smirnov Z		.613	.634	.434	.567	.544
Asymp. Sig. (2-tailed)		.847	.816	.992	.904	.929

a Test distribution is Normal.

b Calculated from data.

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Runs Test

	P-plot1	P-plot2	P-plot3	P-plot4	P-plot5
Test Value(a)	20.34	24.99	17.64	30.12	26.60
Cases < Test Value	1	1	1	1	1
Cases >= Test Value	2	2	2	2	2
Total Cases	3	3	3	3	3
Number of Runs	2	3	2	2	2
Z	.000	.354	.000	.000	.000
Asymp. Sig. (2-tailed)	1.000	.724	1.000	1.000	1.000

a Median

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5. K

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
K-plot1	3	117.0000	4.20357	112.70	121.10
K-plot2	3	98.2333	2.34592	95.60	100.10
K-plot3	3	88.6267	1.24026	87.24	89.63
K-plot4	3	101.5733	.53482	101.17	102.18
K-plot5	3	117.7400	1.36722	116.33	119.06

One-Sample Kolmogorov-Smirnov Test

		K-plot1	K-plot2	K-plot3	K-plot4	K-plot5
N		3	3	3	3	3
Normal Parameters(a,b)	Mean	117.0000	98.2333	88.6267	101.5733	117.7400
	Std. Deviation	4.20357	2.34592	1.24026	.53482	1.36722
Most Extreme Differences	Absolute	.186	.295	.288	.315	.193
	Positive	.180	.213	.209	.315	.182
	Negative	-.186	-.295	-.288	-.225	-.193
Kolmogorov-Smirnov Z		.322	.511	.499	.545	.334
Asymp. Sig. (2-tailed)		1.000	.957	.965	.928	1.000

a Test distribution is Normal.

b Calculated from data.

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Runs Test

	K-plot1	K-plot2	K-plot3	K-plot4	K-plot5
Test Value(a)	117.20	99.00	89.01	101.37	117.83
Cases < Test Value	1	1	1	1	1
Cases >= Test Value	2	2	2	2	2
Total Cases	3	3	3	3	3
Number of Runs	2	2	2	2	3
Z	.000	.000	.000	.000	.354
Asymp. Sig. (2-tailed)	1.000	1.000	1.000	1.000	.724

a Median

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6. N

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
N-plot1	3	.2833	.04509	.24	.33
N-plot2	3	.2333	.00577	.23	.24
N-plot3	3	.2267	.01528	.21	.24
N-plot4	3	.2100	.03000	.18	.24
N-plot5	3	.1967	.02517	.17	.22

One-Sample Kolmogorov-Smirnov Test

		N-plot1	N-plot2	N-plot3	N-plot4	N-plot5
N		3	3	3	3	3
Normal Parameters(a,b)	Mean	.2833	.2333	.2267	.2100	.1967
	Std. Deviation	.04509	.00577	.01528	.03000	.02517
Most Extreme Differences	Absolute	.196	.385	.253	.175	.219
	Positive	.196	.385	.196	.175	.189
	Negative	-.183	-.282	-.253	-.175	-.219
Kolmogorov-Smirnov Z		.340	.667	.438	.303	.380
Asymp. Sig. (2-tailed)		1.000	.766	.991	1.000	.999

a Test distribution is Normal.

b Calculated from data.

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Runs Test

	N-plot1	N-plot2	N-plot3	N-plot4	N-plot5
Test Value(a)	.28	.23	.23	.21	.20
Cases < Test Value	1	1	1	1	1
Cases >= Test Value	2	2	2	2	2
Total Cases	3	3	3	3	3
Number of Runs	3	2	3	2	2
Z	.354	.000	.354	.000	.000
Asymp. Sig. (2-tailed)	.724	1.000	.724	1.000	1.000

a Median

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7. CEC

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
CEC-plot1	3	9.0033	.14503	8.86	9.15
CEC-plot2	3	12.5733	.16503	12.41	12.74
CEC-plot3	3	10.1033	.10504	10.00	10.21
CEC-plot4	3	9.7467	.42501	9.32	10.17
CEC-plot5	3	8.5600	.02000	8.54	8.58

One-Sample Kolmogorov-Smirnov Test

		CEC-plot1	CEC-plot2	CEC-plot3	CEC-plot4	CEC-plot5
N		3	3	3	3	3
Normal Parameters(a,b)	Mean	9.0033	12.5733	10.1033	9.7467	8.5600
	Std. Deviation	.14503	.16503	.10504	.42501	.02000
Most Extreme Differences	Absolute	.177	.177	.179	.176	.175
	Positive	.176	.175	.179	.176	.175
	Negative	-.177	-.177	-.178	-.174	-.175
Kolmogorov-Smirnov Z		.307	.307	.311	.304	.303
Asymp. Sig. (2-tailed)		1.000	1.000	1.000	1.000	1.000

a Test distribution is Normal.

b Calculated from data.

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Runs Test

	CEC-plot1	CEC-plot2	CEC-plot3	CEC-plot4	CEC-plot5
Test Value(a)	9.00	12.57	10.10	9.75	8.56
Cases < Test Value	1	1	1	1	1
Cases >= Test Value	2	2	2	2	2
Total Cases	3	3	3	3	3
Number of Runs	2	2	2	3	3
Z	.000	.000	.000	.354	.354
Asymp. Sig. (2-tailed)	1.000	1.000	1.000	.724	.724

a Median

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8. Total-Zn (TZn)

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
TZn-plot1	3	3123.0038	151.41370	2995.74	3290.46
TZn-plot2	3	1722.2870	97.27692	1622.86	1817.26
TZn-plot3	3	897.5003	66.68399	842.94	971.83
TZn-plot4	3	823.1126	85.55095	739.00	910.03
TZn-plot5	3	302.3097	105.96327	222.46	422.52

One-Sample Kolmogorov-Smirnov Test

		TZn-plot1	TZn-plot2	TZn-plot3	TZn-plot4	TZn-plot5
N		3	3	3	3	3
Normal Parameters(a,b)	Mean	3123.0038	1722.2870	897.5003	823.1126	302.3097
	Std. Deviation	151.413	97.276	66.683	85.550	105.963
Most Extreme Differences	Absolute	.271	.185	.283	.180	.315
	Positive	.271	.180	.283	.180	.315
	Negative	-.200	-.185	-.207	-.179	-.226
Kolmogorov-Smirnov Z		.470	.320	.491	.311	.546
Asymp. Sig. (2-tailed)		.980	1.000	.970	1.000	.927

a Test distribution is Normal.

b Calculated from data.

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Runs Test

	TZn-plot1	TZn-plot2	TZn-plot3	TZn-plot4	TZn-plot5
Test Value(a)	3082.82	1726.75	877.73	820.31	261.95
Cases < Test Value	1	1	1	1	1
Cases >= Test Value	2	2	2	2	2
Total Cases	3	3	3	3	3
Number of Runs	3	3	2	2	3
Z	.354	.354	.000	.000	.354
Asymp. Sig. (2-tailed)	.724	.724	1.000	1.000	.724

a Median

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9. Total-Cd (TCd)

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
TCd-plot1	3	66.0640	2.94133	63.18	69.06
TCd-plot2	3	27.8513	2.85653	24.65	30.14
TCd-plot3	3	10.5030	.23378	10.23	10.66
TCd-plot4	3	8.1893	.90362	7.17	8.90
TCd-plot5	3	5.5547	.97820	4.97	6.68

One-Sample Kolmogorov-Smirnov Test

		TCd-plot1	TCd-plot2	TCd-plot3	TCd-plot4	TCd-plot5
N		3	3	3	3	3
Normal Parameters(a,b)	Mean	66.0640	27.8513	10.5030	8.1893	5.5547
	Std. Deviation	2.94133	2.85653	.23378	.90362	.97820
Most Extreme Differences	Absolute	.180	.293	.355	.298	.379
	Positive	.180	.212	.255	.215	.379
	Negative	-.179	-.293	-.355	-.298	-.276
Kolmogorov-Smirnov Z		.313	.507	.615	.517	.656
Asymp. Sig. (2-tailed)		1.000	.960	.843	.952	.783

a Test distribution is Normal.

b Calculated from data.

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Runs Test

	TCd-plot1	TCd-plot2	TCd-plot3	TCd-plot4	TCd-plot5
Test Value(a)	65.96	28.77	10.62	8.49	5.01
Cases < Test Value	1	1	1	1	1
Cases >= Test Value	2	2	2	2	2
Total Cases	3	3	3	3	3
Number of Runs	2	2	3	2	3
Z	.000	.000	.354	.000	.354
Asymp. Sig. (2-tailed)	1.000	1.000	.724	1.000	.724

a Median

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10. Total-Pb (TPb)

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
TPb-plot1	3	136.8293	3.89557	133.40	141.07
TPb-plot2	3	76.0093	5.09297	70.47	80.50
TPb-plot3	3	44.5537	4.08808	42.05	49.27
TPb-plot4	3	42.5760	7.49299	34.32	48.93
TPb-plot5	3	30.0857	1.20226	28.71	30.92

One-Sample Kolmogorov-Smirnov Test

		TPb-plot1	TPb-plot2	TPb-plot3	TPb-plot4	TPb-plot5
N		3	3	3	3	3
Normal Parameters(a,b)	Mean	136.829	76.009	44.553	42.576	30.085
	Std. Deviation	3.89557	5.09297	4.08808	7.49299	1.20226
Most Extreme Differences	Absolute	.249	.248	.372	.267	.343
	Positive	.249	.195	.372	.198	.245
	Negative	-.195	-.248	-.270	-.267	-.343
Kolmogorov-Smirnov Z		.431	.430	.645	.462	.593
Asymp. Sig. (2-tailed)		.992	.993	.800	.983	.873

a Test distribution is Normal.

b Calculated from data.

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Runs Test

	TPb-plot1	TPb-plot2	TPb-plot3	TPb-plot4	TPb-plot5
Test Value(a)	136.02	77.06	42.35	44.48	30.63
Cases < Test Value	1	1	1	1	1
Cases >= Test Value	2	2	2	2	2
Total Cases	3	3	3	3	3
Number of Runs	2	2	2	2	2
Z	.000	.000	.000	.000	.000
Asymp. Sig. (2-tailed)	1.000	1.000	1.000	1.000	1.000

a Median

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11. Total-Fe (TFe)

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
TFe-plot1	3	18108.4028	72.19607	18041.57	18184.97
TFe-plot2	3	15984.9602	775.40349	15176.64	16722.61
TFe-plot3	3	11527.0383	1038.17132	10626.28	12662.45
TFe-plot4	3	9540.0115	403.57227	9074.94	9798.07
TFe-plot5	3	9070.2194	134.99156	8917.24	9172.61

One-Sample Kolmogorov-Smirnov Test

		TFe-plot1	TFe-plot2	TFe-plot3	TFe-plot4	TFe-plot5
N		3	3	3	3	3
Normal Parameters(a,b)	Mean	18108.4	15984.9	11527.0	9540.0	9070.2
	Std. Deviation	72.19	775.40	1038.17	403.57	134.99
Most Extreme Differences	Absolute	.220	.203	.256	.363	.313
	Positive	.220	.185	.256	.261	.224
	Negative	-.189	-.203	-.196	-.363	-.313
Kolmogorov-Smirnov Z		.382	.352	.444	.628	.542
Asymp. Sig. (2-tailed)		.999	1.000	.989	.825	.931

a Test distribution is Normal.

b Calculated from data.

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Runs Test

	TFe-plot1	TFe-plot2	TFe-plot3	TFe-plot4	TFe-plot5
Test Value(a)	18098.67	16055.63	11292.39	9747.02	9120.81
Cases < Test Value	1	1	1	1	1
Cases >= Test Value	2	2	2	2	2
Total Cases	3	3	3	3	3
Number of Runs	3	3	3	2	3
Z	.354	.354	.354	.000	.354
Asymp. Sig. (2-tailed)	.724	.724	.724	1.000	.724

a Median

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12. DTPA-Zn (DZn)

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
DZn-plot1	3	101.0103	20.77465	78.53	119.50
DZn-plot2	3	65.4613	2.85531	62.68	68.39
DZn-plot3	3	62.6760	21.06770	45.38	86.14
DZn-plot4	3	68.1107	20.18784	51.96	90.74
DZn-plot5	3	98.4490	5.70363	92.13	103.22

One-Sample Kolmogorov-Smirnov Test

		DZn-plot1	DZn-plot2	DZn-plot3	DZn-plot4	DZn-plot5
N		3	3	3	3	3
Normal Parameters(a,b)	Mean	101.010	65.461	62.676	68.110	98.449
	Std. Deviation	20.7746	2.8553	21.0677	20.1878	5.7036
Most Extreme Differences	Absolute	.243	.187	.282	.293	.274
	Positive	.194	.187	.282	.293	.202
	Negative	-.243	-.181	-.206	-.212	-.274
Kolmogorov-Smirnov Z		.421	.324	.488	.507	.474
Asymp. Sig. (2-tailed)		.994	1.000	.971	.959	.978

a Test distribution is Normal.

b Calculated from data.

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Runs Test

	DZn-plot1	DZn-plot2	DZn-plot3	DZn-plot4	DZn-plot5
Test Value(a)	105.01	65.32	56.51	61.63	100.00
Cases < Test Value	1	1	1	1	1
Cases >= Test Value	2	2	2	2	2
Total Cases	3	3	3	3	3
Number of Runs	3	2	2	3	2
Z	.354	.000	.000	.354	.000
Asymp. Sig. (2-tailed)	.724	1.000	1.000	.724	1.000

a Median

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13. DTPA-Cd (DCd)

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
DCd-plot1	3	13.0883	1.50298	11.78	14.73
DCd-plot2	3	3.8310	.16003	3.66	3.98
DCd-plot3	3	8.0063	1.32902	6.57	9.19
DCd-plot4	3	6.6923	.86497	5.74	7.42
DCd-plot5	3	4.6527	.56822	4.20	5.29

One-Sample Kolmogorov-Smirnov Test

		DCd-plot1	DCd-plot2	DCd-plot3	DCd-plot4	DCd-plot5
N		3	3	3	3	3
Normal Parameters(a,b)	Mean	13.0883	3.8310	8.0063	6.6923	4.6527
	Std. Deviation	1.50298	.16003	1.32902	.86497	.56822
Most Extreme Differences	Absolute	.254	.204	.244	.271	.293
	Positive	.254	.185	.194	.200	.293
	Negative	-.196	-.204	-.244	-.271	-.212
Kolmogorov-Smirnov Z		.441	.353	.422	.469	.508
Asymp. Sig. (2-tailed)		.990	1.000	.994	.980	.958

a Test distribution is Normal.

b Calculated from data.

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Runs Test

	DCd-plot1	DCd-plot2	DCd-plot3	DCd-plot4	DCd-plot5
Test Value(a)	12.76	3.85	8.27	6.92	4.47
Cases < Test Value	1	1	1	1	1
Cases >= Test Value	2	2	2	2	2
Total Cases	3	3	3	3	3
Number of Runs	2	2	2	2	3
Z	.000	.000	.000	.000	.354
Asymp. Sig. (2-tailed)	1.000	1.000	1.000	1.000	.724

a Median

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14. DTPA-Pb (DPb)

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
DPb-plot1	3	28.6977	2.43520	26.26	31.13
DPb-plot2	3	28.7698	1.68140	27.09	30.45
DPb-plot3	3	18.8035	.54810	18.26	19.35
DPb-plot4	3	15.1185	1.10145	14.02	16.22
DPb-plot5	3	10.8940	2.59205	8.30	13.49

One-Sample Kolmogorov-Smirnov Test

		DPb-plot1	DPb-plot2	DPb-plot3	DPb-plot4	DPb-plot5
N		3	3	3	3	3
Normal Parameters(a,b)	Mean	28.6977	28.7698	18.8035	15.1185	10.8940
	Std. Deviation	2.43520	1.68140	.54810	1.10145	2.59205
Most Extreme Differences	Absolute	.175	.175	.175	.175	.175
	Positive	.175	.175	.175	.175	.175
	Negative	-.175	-.175	-.175	-.175	-.175
Kolmogorov-Smirnov Z		.303	.303	.303	.303	.303
Asymp. Sig. (2-tailed)		1.000	1.000	1.000	1.000	1.000

a Test distribution is Normal.

b Calculated from data.

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Runs Test

	DPb-plot1	DPb-plot2	DPb-plot3	DPb-plot4	DPb-plot5
Test Value(a)	28.70	28.77	18.80	15.12	10.89
Cases < Test Value	1	1	1	1	1
Cases >= Test Value	2	2	2	2	2
Total Cases	3	3	3	3	3
Number of Runs	2	2	2	2	2
Z	.000	.000	.000	.000	.000
Asymp. Sig. (2-tailed)	1.000	1.000	1.000	1.000	1.000

a Median

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15. DTPA-Fe (DFe)

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
DFe-plot1	3	56.7337	.67940	56.05	57.41
DFe-plot2	3	38.0307	1.78240	36.25	39.81
DFe-plot3	3	64.4787	.86780	63.61	65.35
DFe-plot4	3	104.1820	1.81030	102.37	105.99
DFe-plot5	3	54.9888	10.69540	44.29	65.68

One-Sample Kolmogorov-Smirnov Test

		DFe-plot1	DFe-plot2	DFe-plot3	DFe-plot4	DFe-plot5
N		3	3	3	3	3
Normal Parameters(a,b)	Mean	56.733	38.030	64.478	104.182	54.988
	Std. Deviation	.6794	1.7824	.8678	1.8103	10.6954
Most Extreme Differences	Absolute	.175	.175	.175	.175	.175
	Positive	.175	.175	.175	.175	.175
	Negative	-.175	-.175	-.175	-.175	-.175
Kolmogorov-Smirnov Z		.303	.303	.303	.303	.303
Asymp. Sig. (2-tailed)		1.000	1.000	1.000	1.000	1.000

a Test distribution is Normal.

b Calculated from data.

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Runs Test

	DFe-plot1	DFe-plot2	DFe-plot3	DFe-plot4	DFe-plot5
Test Value(a)	56.73	38.03	64.48	104.18	54.99
Cases < Test Value	1	1	1	1	1
Cases >= Test Value	2	2	2	2	2
Total Cases	3	3	3	3	3
Number of Runs	2	2	2	2	2
Z	.000	.000	.000	.000	.000
Asymp. Sig. (2-tailed)	1.000	1.000	1.000	1.000	1.000

a Median

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16. Moisture

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Moisture-plot1	3	1.9267	.09504	1.83	2.02
Moisture-plot2	3	1.9500	.11533	1.82	2.04
Moisture-plot3	3	1.7333	.23159	1.54	1.99
Moisture-plot4	3	1.7667	.09504	1.67	1.86
Moisture-plot5	3	1.7967	.12897	1.69	1.94

One-Sample Kolmogorov-Smirnov Test

		Moisture-plot1	Moisture-plot2	Moisture-plot3	Moisture-plot4	Moisture-plot5
N		3	3	3	3	3
Normal Parameters(a,b)	Mean	1.9267	1.9500	1.7333	1.7667	1.7967
	Std. Deviation	.09504	.11533	.23159	.09504	.12897
Most Extreme Differences	Absolute	.181	.302	.274	.181	.279
	Positive	.179	.218	.274	.179	.279
	Negative	-.181	-.302	-.202	-.181	-.204
Kolmogorov-Smirnov Z		.313	.524	.475	.313	.483
Asymp. Sig. (2-tailed)		1.000	.947	.978	1.000	.974

a Test distribution is Normal.

b Calculated from data.

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Runs Test

	Moisture-plot1	Moisture-plot2	Moisture-plot3	Moisture-plot4	Moisture-plot5
Test Value(a)	1.93	1.99	1.67	1.77	1.76
Cases < Test Value	1	1	1	1	1
Cases >= Test Value	2	2	2	2	2
Total Cases	3	3	3	3	3
Number of Runs	3	3	3	2	3
Z	.354	.354	.354	.000	.354
Asymp. Sig. (2-tailed)	.724	.724	.724	1.000	.724

a Median

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17. CFU

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
CFU-plot1	3	6.9600	.16703	6.78	7.11
CFU-plot2	3	7.0267	.03512	6.99	7.06
CFU-plot3	3	6.8333	.19655	6.61	6.98
CFU-plot4	3	6.9767	.05508	6.92	7.03
CFU-plot5	3	6.9467	.06110	6.88	7.00

One-Sample Kolmogorov-Smirnov Test

		CFU-plot1	CFU-plot2	CFU-plot3	CFU-plot4	CFU-plot5
N		3	3	3	3	3
Normal Parameters(a,b)	Mean	6.9600	7.0267	6.8333	6.9767	6.9467
	Std. Deviation	.16703	.03512	.19655	.05508	.06110
Most Extreme Differences	Absolute	.238	.204	.318	.191	.253
	Positive	.193	.185	.228	.182	.196
	Negative	-.238	-.204	-.318	-.191	-.253
Kolmogorov-Smirnov Z		.412	.354	.552	.330	.438
Asymp. Sig. (2-tailed)		.996	1.000	.921	1.000	.991

a Test distribution is Normal.

b Calculated from data.

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Runs Test

	CFU-plot1	CFU-plot2	CFU-plot3	CFU-plot4	CFU-plot5
Test Value(a)	6.99	7.03	6.91	6.98	6.96
Cases < Test Value	1	1	1	1	1
Cases >= Test Value	2	2	2	2	2
Total Cases	3	3	3	3	3
Number of Runs	2	2	2	3	3
Z	.000	.000	.000	.354	.354
Asymp. Sig. (2-tailed)	1.000	1.000	1.000	.724	.724

a Median

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18. Richness (Rich)

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Rich-plot1	3	12.3333	1.52753	11.00	14.00
Rich-plot2	3	12.6667	1.52753	11.00	14.00
Rich-plot3	3	11.0000	1.73205	9.00	12.00
Rich-plot4	3	12.0000	1.00000	11.00	13.00
Rich-plot5	3	12.6667	2.08167	11.00	15.00

One-Sample Kolmogorov-Smirnov Test

		Rich-plot1	Rich-plot2	Rich-plot3	Rich-plot4	Rich-plot5
N		3	3	3	3	3
Normal Parameters(a,b)	Mean	12.3333	12.6667	11.0000	12.0000	12.6667
	Std. Deviation	1.52753	1.52753	1.73205	1.00000	2.08167
Most Extreme Differences	Absolute	.253	.253	.385	.175	.292
	Positive	.253	.196	.282	.175	.292
	Negative	-.196	-.253	-.385	-.175	-.212
Kolmogorov-Smirnov Z		.438	.438	.667	.303	.506
Asymp. Sig. (2-tailed)		.991	.991	.766	1.000	.960

a Test distribution is Normal.

b Calculated from data.

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Runs Test

	Rich-plot1	Rich-plot2	Rich-plot3	Rich-plot4	Rich-plot5
Test Value(a)	12.00	13.00	12.00	12.00	12.00
Cases < Test Value	1	1	1	1	1
Cases >= Test Value	2	2	2	2	2
Total Cases	3	3	3	3	3
Number of Runs	3	3	2	3	2
Z	.354	.354	.000	.354	.000
Asymp. Sig. (2-tailed)	.724	.724	1.000	.724	1.000

a Median

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19. Evenness (Even)

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Even-plot1	3	.8667	.04726	.83	.92
Even-plot2	3	.7567	.03512	.72	.79
Even-plot3	3	.8233	.06807	.77	.90
Even-plot4	3	.7500	.06000	.69	.81
Even-plot5	3	.7433	.10693	.62	.81

One-Sample Kolmogorov-Smirnov Test

		Even-plot1	Even-plot2	Even-plot3	Even-plot4	Even-plot5
N		3	3	3	3	3
Normal Parameters(a,b)	Mean	.8667	.7567	.8233	.7500	.7433
	Std. Deviation	.04726	.03512	.06807	.06000	.10693
Most Extreme Differences	Absolute	.304	.204	.301	.175	.369
	Positive	.304	.185	.301	.175	.266
	Negative	-.219	-.204	-.217	-.175	-.369
Kolmogorov-Smirnov Z		.527	.354	.521	.303	.638
Asymp. Sig. (2-tailed)		.944	1.000	.949	1.000	.810

a Test distribution is Normal.

b Calculated from data.

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Runs Test

	Even-plot1	Even-plot2	Even-plot3	Even-plot4	Even-plot5
Test Value(a)	.85	.76	.80	.75	.80
Cases < Test Value	1	1	1	1	1
Cases >= Test Value	2	2	2	2	2
Total Cases	3	3	3	3	3
Number of Runs	2	2	3	2	2
Z	.000	.000	.354	.000	.000
Asymp. Sig. (2-tailed)	1.000	1.000	.724	1.000	1.000

a Median

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20. Shannon-Weiner index (Shannon)

Descriptive Statistics

	N	Mean	Std. Deviation	Minimum	Maximum
Shannon-plot1	3	2.1700	.11790	2.04	2.27
Shannon-plot2	3	1.9133	.04041	1.89	1.96
Shannon-plot3	3	1.9633	.03786	1.92	1.99
Shannon-plot4	3	1.8600	.09000	1.77	1.95
Shannon-plot5	3	1.8900	.34598	1.50	2.16

One-Sample Kolmogorov-Smirnov Test

		Shannon -plot1	Shannon -plot2	Shannon -plot3	Shannon -plot4	Shannon -plot5
N		3	3	3	3	3
Normal Parameters(a,b)	Mean	2.1700	1.9133	1.9633	1.8600	1.8900
	Std. Deviation	.11790	.04041	.03786	.09000	.34598
Most Extreme Differences	Absolute	.267	.385	.337	.175	.302
	Positive	.198	.385	.241	.175	.218
	Negative	-.267	-.282	-.337	-.175	-.302
Kolmogorov-Smirnov Z		.463	.667	.583	.303	.524
Asymp. Sig. (2-tailed)		.983	.766	.886	1.000	.947

a Test distribution is Normal.

b Calculated from data.

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Runs Test

	Shannon-plot1	Shannon-plot2	Shannon-plot3	Shannon-plot4	Shannon-plot5
Test Value(a)	2.20	1.89	1.98	1.86	2.01
Cases < Test Value	1	1	1	1	1
Cases >= Test Value	2	2	2	2	2
Total Cases	3	3	3	3	3
Number of Runs	3	2	3	2	2
Z	.354	.000	.354	.000	.000
Asymp. Sig. (2-tailed)	.724	1.000	.724	1.000	1.000

a Median

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Appendix-C-2 Chemical properties and bacterial diversity of soil collected from the field site at VE stage of maize growth

		Sum of Squares	df	Mean Square	F	Sig.
pH	Between Groups	2.652	4	.663	53.705	.000
	Within Groups	.123	10	.012		
	Total	2.776	14			
Ec	Between Groups	35209.936	4	8802.484	66.538	.000
	Within Groups	1322.933	10	132.293		
	Total	36532.869	14			
Om	Between Groups	19.083	4	4.771	14.743	.000
	Within Groups	3.236	10	.324		
	Total	22.319	14			
P	Between Groups	300.021	4	75.005	1117.704	.000
	Within Groups	.671	10	.067		
	Total	300.693	14			
K	Between Groups	1893.768	4	473.442	88.109	.000
	Within Groups	53.734	10	5.373		
	Total	1947.502	14			

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		Sum of Squares	df	Mean Square	F	Sig.
N	Between Groups	.013	4	.003	4.283	.028
	Within Groups	.008	10	.001		
	Total	.021	14			
CEC	Between Groups	29.291	4	7.323	152.348	.000
	Within Groups	.481	10	.048		
	Total	29.772	14			
Moisture	Between Groups	.114	4	.029	1.406	.301
	Within Groups	.203	10	.020		
	Total	.318	14			
Total-Zn	Between Groups	14578099.153	4	3644524.788	329.030	.000
	Within Groups	110765.684	10	11076.568		
	Total	14688864.838	14			
Total -Cd	Between Groups	7667.742	4	1916.935	514.222	.000
	Within Groups	37.278	10	3.728		
	Total	7705.020	14			
Total -Pb	Between Groups	22246.347	4	5561.587	240.935	.000
	Within Groups	230.833	10	23.083		
	Total	22477.180	14			

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		Sum of Squares	df	Mean Square	F	Sig.
Total -Fe	Between Groups	193415078.349	4	48353769.587	129.610	.000
	Within Groups	3730711.656	10	373071.166		
	Total	197145790.005	14			
DTPA-Zn	Between Groups	4292.893	4	1073.223	4.054	.033
	Within Groups	2647.335	10	264.733		
	Total	6940.228	14			
DTPA-Cd	Between Groups	160.214	4	40.054	39.100	.000
	Within Groups	10.244	10	1.024		
	Total	170.458	14			
DTPA-Pb	Between Groups	779.086	4	194.772	57.321	.000
	Within Groups	33.979	10	3.398		
	Total	813.065	14			
DTPA-Fe	Between Groups	7268.174	4	1817.043	74.432	.000
	Within Groups	244.121	10	24.412		
	Total	7512.294	14			
CFU	Between Groups	.034	4	.009	.815	.544
	Within Groups	.105	10	.010		
	Total	.139	14			

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		Sum of Squares	df	Mean Square	F	Sig.
Evenness	Between Groups	.036	4	.009	1.922	.183
	Within Groups	.046	10	.005		
	Total	.082	14			
Richness	Between Groups	5.733	4	1.433	.551	.703
	Within Groups	26.000	10	2.600		
	Total	31.733	14			
Shannon	Between Groups	.184	4	.046	1.585	.252
	Within Groups	.290	10	.029		
	Total	.473	14			

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Appendix C-3 Effect of Zn/Cd/Pb contaminated soil on maize growth in pot experiment

1. Control

		Sum of Squares	df	Mean Square	F	Sig.
L-Chlorophyll	Between Groups	320.827	3	106.942	124.351	.000
	Within Groups	6.880	8	.860		
	Total	327.707	11			
U-Chlorophyll	Between Groups	410.596	3	136.865	77.617	.000
	Within Groups	14.107	8	1.763		
	Total	424.703	11			
Shoot-Dry	Between Groups	.299	3	.100	99.511	.000
	Within Groups	.008	8	.001		
	Total	.307	11			
Height	Between Groups	880.667	3	293.556	320.242	.000
	Within Groups	7.333	8	.917		
	Total	888.000	11			

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2. Treatment with low of Zn/Cd/Pb concentrations

		Sum of Squares	df	Mean Square	F	Sig.
L-Chlorophyll	Between Groups	302.873	3	100.958	56.931	.000
	Within Groups	14.187	8	1.773		
	Total	317.060	11			
U-Chlorophyll	Between Groups	218.777	3	72.926	109.388	.000
	Within Groups	5.333	8	.667		
	Total	224.110	11			
Shoot-Dry	Between Groups	.418	3	.139	290.101	.000
	Within Groups	.004	8	.000		
	Total	.422	11			
Height	Between Groups	797.583	3	265.861	455.762	.000
	Within Groups	4.667	8	.583		
	Total	802.250	11			

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3. Treatment with medium of Zn/Cd/Pb concentrations

		Sum of Squares	df	Mean Square	F	Sig.
L-Chlorophyll	Between Groups	156.810	3	52.270	41.130	.000
	Within Groups	10.167	8	1.271		
	Total	166.977	11			
U-Chlorophyll	Between Groups	249.803	3	83.268	979.618	.000
	Within Groups	.680	8	.085		
	Total	250.483	11			
Shoot-Dry	Between Groups	.332	3	.111	215.461	.000
	Within Groups	.004	8	.001		
	Total	.336	11			
Height	Between Groups	1096.667	3	365.556	548.333	.000
	Within Groups	5.333	8	.667		
	Total	1102.000	11			

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4. Treatment with high of Zn/Cd/Pb concentrations

		Sum of Squares	df	Mean Square	F	Sig.
L-Chlorophyll	Between Groups	41.710	3	13.903	2.703	.116
	Within Groups	41.147	8	5.143		
	Total	82.857	11			
U-Chlorophyll	Between Groups	205.516	3	68.505	18.436	.001
	Within Groups	29.727	8	3.716		
	Total	235.243	11			
Shoot-Dry	Between Groups	.398	3	.133	207.249	.000
	Within Groups	.005	8	.001		
	Total	.403	11			
Height	Between Groups	1495.000	3	498.333	373.750	.000
	Within Groups	10.667	8	1.333		
	Total	1505.667	11			

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5. Maize plant growth after 1 week

		Sum of Squares	df	Mean Square	F	Sig.
L-Chlorophyll	Between Groups	181.603	3	60.534	34.509	.000
	Within Groups	14.033	8	1.754		
	Total	195.637	11			
U-Chlorophyll	Between Groups	12.803	3	4.268	.861	.500
	Within Groups	39.653	8	4.957		
	Total	52.457	11			
Shoot-Dry	Between Groups	.005	3	.002	38.368	.000
	Within Groups	.000	8	.000		
	Total	.005	11			
Height	Between Groups	104.250	3	34.750	23.167	.000
	Within Groups	12.000	8	1.500		
	Total	116.250	11			

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6. Maize plant growth after 2 week

		Sum of Squares	df	Mean Square	F	Sig.
L-Chlorophyll	Between Groups	91.257	3	30.419	6.198	.018
	Within Groups	39.260	8	4.908		
	Total	130.517	11			
U-Chlorophyll	Between Groups	71.829	3	23.943	32.799	.000
	Within Groups	5.840	8	.730		
	Total	77.669	11			
Shoot-Dry	Between Groups	.002	3	.001	2.324	.151
	Within Groups	.003	8	.000		
	Total	.005	11			
Height	Between Groups	18.917	3	6.306	25.222	.000
	Within Groups	2.000	8	.250		
	Total	20.917	11			

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7. Maize plant growth after 3 week

		Sum of Squares	df	Mean Square	F	Sig.
L-Chlorophyll	Between Groups	21.623	3	7.208	4.658	.036
	Within Groups	12.380	8	1.548		
	Total	34.003	11			
U-Chlorophyll	Between Groups	166.909	3	55.636	143.578	.000
	Within Groups	3.100	8	.388		
	Total	170.009	11			
Shoot-Dry	Between Groups	.033	3	.011	42.893	.000
	Within Groups	.002	8	.000		
	Total	.035	11			
Height	Between Groups	2.667	3	.889	.711	.572
	Within Groups	10.000	8	1.250		
	Total	12.667	11			

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8. Maize plant growth after 4 week

		Sum of Squares	df	Mean Square	F	Sig.
L-Chlorophyll	Between Groups	129.383	3	43.128	51.444	.000
	Within Groups	6.707	8	.838		
	Total	136.089	11			
U-Chlorophyll	Between Groups	104.976	3	34.992	223.353	.000
	Within Groups	1.253	8	.157		
	Total	106.229	11			
Shoot-Dry	Between Groups	.021	3	.007	3.518	.069
	Within Groups	.016	8	.002		
	Total	.037	11			
Height	Between Groups	104.250	3	34.750	69.500	.000
	Within Groups	4.000	8	.500		
	Total	108.250	11			

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BIOGRAPHY

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EDUCATION

2011-2017	Ph.D. (Biology)	Mahasarakham University, Thailand
2008-2011	M.Sc. (Biology)	Mahasarakham University, Thailand
2004-2007	B.Eng (Chemical)	King Mongkut's University of Technology Thonburi

SCHOLARSHIPS & AWARDS

Junior Science Talent Project (JSTP), National Science and Technology Development Agency (NSTDA), Thailand (duration: 2003-2017).

The Student Travel Award of ICOBTE 2015, Received for 13th International Conference on the Biogeochemistry of Trace Elements (ICOBTE 2015), Fukuoka international congress center, Japan, 12-16 July 2015

World Bank Grant, Received for 22nd Annual Conference of The International Environmetrics Society (TIES 2012), University of Hyderabad, Hyderabad, India, 3-6 January 2012

The Professor Dr. Tab Nilanidhi Foundation Award, Certificate of Academic Excellence from the Professor Dr. Tab Nilanidhi Foundation, Thailand, 21 August 2010

The 2nd team of Thailand, Elected for a project of Japan Aerospace Exploration Agency (JAXA). *"Investigation of physiology and biochemistry change under the zero gravity for Biophytum adiantoides Wight ex Edgew. & Hook. f. "*

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