DOCTOR OF PHILOSOPHY

The potential for wheat roots in phytoremediation of phenolic compounds

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Award date:
2016

Awarding institution:
Bangor University

The potential for wheat roots in phytoremediation of phenolic compounds

A Thesis Submitted to Bangor University

by

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In candidature for the degree of Philosophiae Doctoriae

The School of Biological Science

Bangor University

PRIFYSGOL
BANGOR
UNIVERSITY

June 2016

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Dedication

To my children, Hassan, Rayan, Kadi and Rodena.

Acknowledgement

First of all, I have to thank Allah.

I am grateful to the Government of Saudi Arabia for giving me the opportunity to study in Bangor University. I very grateful to my supervisor Prof Deri Tomos, for his sincere help, advice, guidance and encouragement during the period of study.

I thank Prof Chris Freeman, Dr Hussain Omed and Dr Anil Shirsat from the Internal Supervisory Committee for their valuable recommendation and guidance. Farther thanks go to all my colleagues in the School of Biological Science for their help in the practical aspects and statistical analysis.

Finally, I would like to express my gratitude to my family, specially my husband and my children for providing me with the appropriate conditions, encouragement and support for completions of my thesis.

Abstract

Soil pollution is a global problem, resulting in a major international research effort using bioremediation technology to exploit plant and microorganism in the removal of contaminants. Phenolic compounds are major pollutants from industrial effluents and consequently are present in many soil and water systems throughout the world.

Phytoremediation studies in soil contaminated with phenolic compounds are a challenge. The rhizosphere is an extremely dynamic zone, both spatially and temporally. In order to understand the complex rhizoremediation processes (including root-microbe reactions), there is a need to study the biophysical interactions at the root/soil interface. However, this is limited by sampling and analysis techniques. A modified (SiCSA) Single Cell Sampling and Analysis technique using fine glass microcapillaries was used in an attempt to overcome this issue. This micro-scale technique was used to quantify the polar phenolic compounds such as syringic acid. Phenols of lower polarity offer different technical challenges as they rapidly dissolve in paraffin oil, which was used to prevent the evaporation of micro samples. As a result, a conventional 'macro' approach was also used.

A microcosm system for plant growth was used to facilitate access to soil and roots. The phenolic compounds were analysis using Capillary Zone Electrophoresis (CZE).

The two selected plants, rye and wheat, have the ability to speed up the removal of two selected phenolic compounds from soil after their addition. The pathways through the plant of phenol (an artificial compound) and syringic acid (a biological compound) are different. Different strategies of phytoremediation of these phenolic compounds in soil was demonstrated in wheat. Phenol was absorbed into the root and transported to the leaf. Phenol seemed to be partially accumulated in the leaf, while some amount evaporated through stomata to the atmosphere. Syringic acid was taken up by the root and seemed to be metabolized there within less 2 hours. No evidence was found that this compound is rapidly transported to the leaf. Phytoremediation occurs in ways previously reported, such as metabolism, accumulation and evaporation.

In conclusion, although microbial processes probably dominate the removal from soil of phenolics studied, both rye and wheat behaviour contributed to the removal of phenolic compounds. This indicates the potential of using them in phytoremediation.

Contents

Abbreviations

DDT	Dichlorodiphenyltrichloroethane
PCBs	Polichlorinatedbiphenyls
PAHs	Polycyclic aromatic hydrocarbons
PBDEs	Polybrominated diphenyl ethers
PBBs	Polybrominated biphenyls
TNP	2, 4, 6- Trinitrophenol
TNT	2, 4, 6-Trinitrotoluene
EEA	European Environmental Agency
ESCWA	Economic and Social Commission for Western Asia
UNEP	United National Environment Programme
WHO	World Health Organization
4-C-2-FP	4-chloro-2-fluorophenol
NMR	Nuclear magnetic resonance
BPA	Bisphenol A
PCP	Pentachlorophenol
2, 4-DNP	2, 4-Dinitrophenol
CT	X-ray Computed Tomography
MRI	Magnetic Resonance Imaging
SiCSA	Single Cell Sampling and Analysis
DU	Uranium (depleted)
CZE	Capillary zone electrophoresis
$Na_2B_4O_7$,	Sodium Tetraborate

KH_2PO_4	Potassium Dihydrogen Phosphate
CH_3CN	Acetonitrile
P	Phenol
2CP	2-Chlorophenol
3CP	3-Chlorophenol
DMAP	2, 5-Dimethoxy-4-acetophenone
LDOPA	L-3, 4-dihydroxy phenylalanine
H_2O_2	Hydrogen peroxide
K_{OW}	Partition coefficient (octanol/water)
nl	Nanoliter
pl	Picoliter

Figures

Tables

Plates

Chapter 1: Introduction and Literature Review

1.1 Pollution as a global problem

Some of the development projects of our times, are accompanied by environmental degradation. Pollution is an undesirable change in the physical, chemical and biological characteristics of the land, air or water that harmfully effect humans as well as other living plants and animals. Human activity disturbs the natural cycles and results in environmental pollution detrimental to human health and well being. Amongst the activities which contribute to pollution are mining and smelting metals, fossil fuel burning, use of pesticides and fertilizers, production of metal products like batteries, sewage sludge and municipal waste (Chibuike & Obiora, 2014).

Petrochemical industries, oil spills and the Gulf war produced a tremendous amount of pollution to air, soil and water (Das & Chandran, 2010). Kuwait, following destruction of oil producing facilities, had more than 600 oil wells set on fire, causing release of huge quantities of crude oil (Yateem, 2013).

1.2 Soil pollution as a global problem

Over the last decades, the pollution of soil and groundwater is considered to be a serious global problem. It has been attracting the concern and attention of the global community due to the acute increase of soil and water pollution by different types of hazardous chemicals on a global scale. Amongst the major ones are pesticides (Arias-Estévez *et al*.,2008) and other organic compounds (Kukučka *et al*.,2009), heavy metals and salts (Pastor & Hernández ,2012; Chibuike & Obiora, 2014), which have a negative effect on organisms and soil quality (Alkorta & Garbisu, 2001; Ali, 2010; Azaizeh *et al*., 2011 ; Couto *et al* ., 2010). These pollutants / chemicals not only show reduction in plant growth, performance and yield; they also find their way into human systems and cause illness.

The early history of anthropogenic metal pollution has been traced back about eight thousand years at the Faynan Orefield in Jordan. This is considered one of the oldest and longest used sites for copper ore extraction and smelting in the Old World. Studies have shown that removal of metal pollution is achieved only very slowly by natural process (Grattan et *al*., 2007). Toxic

compounds come from human activities or industrialization, and present risks to ecosystems, human health and the environment. Forton *et al*. (2012) and Singer & Warkentin (1996) reported that soil science has played a huge role in understanding and modeling the movement of contaminants throughout soil and water for treatment of environmental problems.

Consequently, the environmental pollution by chemical compounds is widely studied. For example, in Ireland, the measurement of different concentrations of organochlorine insecticide residues and trace elements was studied in agricultural soil, industrial and urban areas. It was found that there were high concentrations of DDT residues, lead and mercury in the soils of town gardens (McGrath, 1995). On the other hand, pesticides and weed killers are very important in food crop production for the elimination of pests and weeds. There is a need to examine and study the fate of these pollutants in the environment due to the detrimental effects and their ability to navigate from the soil to food, water or air. The duration of time the pesticides and pollutants remain in the soil depends on the nature of the material, its strength, components of soil and environmental conditions. By understanding these compounds' nature, interaction with environment and ultimately their fate in environment, humans will be obliged to limit their use. Consequently, environment risk will be reduced and new alternative techniques could be applied (Biro *et al*., 2012). Grabowska (2010) reported that polychlorinated biphenyls (PCBs) are persistent organic contaminants in Poland. Poland, a European Union member and a signatory of international agreements concerning environmental protection, is demanding the statutory control of the use of these compounds.

The contaminants of soil have been represented by different species of chemical such as organochlorine pesticides. Man may be exposed to different toxic compounds in different ways. Plants and grazing animals may help transfer them to the food chain, and this constitutes a threat to human health (Zeliger, 2008). In China, another source of soil pollution has been investigated. It comes from the activities of waste recycling. The risk of these pollutants is illustrated by their existence at a high concentration in human blood and hair; believed to have entered humans through consumption of rice (Zhang *et al.*, 2012).

In a European context, the samples of soils from different sites of landfills in Madrid, Spain, were examined. These sites were covered by soil layers 20 years ago, and the study aimed to assess the effect such human waste had to environmental pollution. A high concentration of several contaminants such as heavy metals, salts and organic compounds was found in the examined soil. These results reflect the ability of certain contaminant compounds or pollutants to persist by resisting natural degradation process over long periods of time. Increased health hazard by exposure to these toxics compounds on human, animals and ecosystems has also been reported (Pastor & Hernández, 2012).

1.3 Industrialization effect on environment and human health

Many toxic compounds have been produced by human activity in industrial or everyday life (Donlon & Bauder, 2006). Despite the benefits of rapid industrialization, it has many drawbacks. It produces hazards which accumulate in the environment (Chen *et al*., 2012; Forton *et al*., 2012; Prabhakar *et al*., 2012). Consequently, many attempts have been made to face this problem. An EEA (2011) report illuminates the damage value of industrial facilities in Europe. Examples of organic toxins are benzene, polycyclic aromatic hydrocarbons (PAHs), dioxins and furans which are emitted from the combustion of fuel and as a result of different industrial processes. They can contribute to bioaccumulation in soil. They are also human carcinogens and, as such, represent a serious environmental problem (Wong, 1987; Liang *et al*., 2012). Industrialization provides a comfortable life for humans, while the industrial wastes destroy the environment. Different biotechnology methods have been used to study the degradation of contaminants (Ramos *et al*., 2005). Amongst the major pollutants are polynitroaromatic compounds (Peres & Agathos,2000) such as, 2, 4, 6- trinitrophenol (TNP) (Hirooka *et al*. ,2006) and 2,4,6-trinitrotoluene (TNT) (Esteve-Nunez *et al*., 2001).They also have the ability to resists the natural biodegradation process (Esteve-Nunez *et al*., 2001; Robertson & Jjemba, 2005).

The report of ESCWA / UNEP by El Raey (2006) describes an investigation on the quality of air and the pollution of atmosphere in a number of different Arab regions. This report aimed to determine how air pollution affects the economy, human health and ecosystem in the Middle East and North Africa countries. The main sources of pollution in these areas; dust and sandstorms, greenhouse gases or different industries have been described. Lastly, the environment has been damaged by hazardous chemicals that come from the waste of

industrialization and agriculture. Furthermore, there are many other reasons behind environment pollution in this century to take into consideration. These include climate change, political problems, industry development and war (Keller,1992;Parry *et al*.,1990).The lack of fresh water and loss of land for cultivation also have been demonstrated (Drake ,1997 ; El-Raey ,1997; Abahussain *et al*.,2002 ; Vicente-Serrano *et al*., 2012). In Egypt, for example, the vast majority of the 20,000 to 50,000 tons of hazardous and clinical wastes comes from industry and hospitals (El-Din El-Dars, 2007). These are incinerated and disposed of into lakes or other special zones. These facts could reflect the extent of danger to human health especially to people who often collect or pick up these wastes (Berman& Wandersman, 1990). In recent years, considerable efforts have been directed to control contamination by industries by means of enhancing the standards of enforcement and monitoring of industrialization (Abd El-Salam, 2010; Hamed & El Mahgary 2004; Hosny & El-Zarka, 2004; Khalil, 2004). In addition, the River Nile quality has been widely studied and monitored (Fishar & Williams 2008; Kassem & Shady, 1996 ; Osman *et al*., 2012)

In the recent report issued by WHO (2011), Saudi Arabia is considered one of the world countries with the most contamination by oil and gas. The pollution level in Saudi Arabia is 143 $\mu g/m^3$. This level is attributed to Saudi Arabia being the highest exporter of petroleum and petroleum-based products to all over the world. Saudi Arabia also has nearly one-fifth of the oil reserves in the world. Consequently, the booming industry has polluted approximately 2,175-miles of Saudi Arabia's coastline. Meanwhile, hydrocarbon contaminants formed 50% the air pollution causing respiratory diseases. The Saudi government intensified efforts to remove the environmental contamination in different ways. French engineering group Alstom has been appointed to monitor and decrease carbon emissions from power plants in industrial cities in Saudi Arabia. It also started using solar power plant to save about 28,000 barrels of diesel fuel per year (WHO, 2011).

Heavy metals are another important source of soil pollution. They are introduced into the soil as waste from industrial processes or agriculture and left without treatment. Therefore, this environmental problem has attracted the attention of many researchers over the last decade. The agriculture soil that is close to the roads, near motor ways, are polluted by heavy metal (Ghaedi, 2006; Li *et al*., 2011).

Soil samples from different sites in Jeddah city in Saudi Arabia were examined to evaluate the influence of heavy traffic on soil content. The study found that lead and zinc were the most common pollutants produced from traffic conditions. Efforts continue to evaluate and control the high concentration of metals in soil (Kadi, 2009).

1.4 Oil spill as a source of pollution

Oil spill is one of the main sources of pollution in the environment because of its immediate and long-term impacts. A number of studies have demonstrated that there is a need for knowledge about the effect of oil spill and its repercussions. The transport of oil by sea currently amounts to more than 100 million tonnes per day with approximately 4000 tankers including nearly 400 million tonnes deadweight on the high seas. Accidents are likely to happen. In case of oil spillage, the extend of pollution is dependent on the oil-spot size on the sea and intertidal ecosystem (Stevens *et al*., 2012). Major oil fields are located in the Eastern region of Saudi Arabia. The Ghawar oil field is part of the largest oil reserve in the world. Several industrial cities are also found in this region and petroleum is shipped all over the world from the port of RasTanura (Din, 1990; Gao *et al*., 2009). Accidental spillage and industrial waste that lead to increased risks of environmental pollution are inevitable. In neighbouring states, conflict is also an issue.

The largest oil spill in history happened during the Gulf War in February 1991, when a massive amount of hydrocarbon was leaked into the marine environment directly (when approximately 730 oil wells were destroyed) or as a result of fall-out from the oil fires. 10.8 million barrels of oil were deposited on the shorelines of Kuwait and Saudi Arabia. The impacts on the environment were predicted to be long term (Saenger, 1994; Al-Thukair *et al*., 2007; Yateem 2013). In 1954, the vessel S.S. Jacob Luckenbach sank in the Farallones Gulf located in central of California. This accident led to the death of many sea animals. This triggered the investigation effect of oil pollution on the sea and its organisms (Hampton *et al*., 2003). More sources of oil spill accidents between1970 to 2004 have been investigated, each reporting similar concerns to the environment and its animals (Burgherr, 2007). In addition, in the UK alone, it is estimated that over one million tonnes of oil are spilled into terrestrial environment every year (Shahsavari *et al*., 2015).

1.5 Phenol and its derivatives are considered to be some of the most dangerous compounds in the soil

Rapid industrialization benefits humans, but it has tremendous and dangerous impact on the environment. Phenolic compounds are universally utilized and widely seen in wastewaters as contaminants. Such phenolic compounds are present in high concentration in industrial wastes as a result of manufacturing processes, especially in the effluents of oil-refineries, petrochemical, paper mills, pesticide manufacturing and coke plants for iron-smelting, food or resin manufacturing industries (Satsangee & Ghosh, 1990; Basha *et al*., 2010). Hence, in many soil and water systems throughout the world, phenol and its derivatives are major presented pollutants from industrial effluents (Gonzalez *et al*., 2013 ; Jha *et al*., 2013).

Phenolic compounds are considered dangerous to the natural and domestic environment. Their danger is often due to their ability to resist natural biodegradation rather than their high concentrations (Siedlecka & Stepnowski, 2005). In addition, due to their extensive usage despite their toxic effects, investigation of these compounds has increased in the recent years (Moyo *et al.*, 2012; Wang *et al.*, 2007; Basha *et al*., 2010). Bacteriological populations are usually reduced and their growth is prevented by phenol or its derivatives. As a result, microbes cannot function in soil or play a role in interaction with plants, in order to change the toxic properties such as high immovability and solubility. They make biological treatment and removal very hard. Therefore, they persist in the environment. Phenol and its derivatives have been considered one of the most resistant and persistent organic chemicals in the environment (Siedlecka & Stepnowski, 2005).

Recently, it has been reported that the accumulation of phenol and its derivatives are a serious environmental problem facing the global community, because they are massively used as essential materials in industry for synthetic polymers and fibres (Karim & Fakhruddin, 2012). Phenolic compounds are also widely produced as waste by various other human activities such as the agricultural or industrial sector. Due to their high toxicity, they are extremely dangerous to living systems (Gianfreda *et al.*, 2006).

A massive amount of solid and liquid wastes is produced by olive oil manufacturing (Caputo *et al*., 2013). Phenolic compounds are released during processing of olive (Rodis *et al*., 2002). The olive oil has only 1 - 2% of phenolics of the total amount in the olive fruit. The rest is lost with wastewater and the pomace by approximately 45% and 53% respectively (Rodis *et al*., 2002). According to Caputo *et al*. (2013), the disposal of waste produced after extraction of

olive oil including phenolic compounds is reported to cause serious pollution to soil and ground water. This may attribute change in soil chemical and physical properties and further inhibit the microbial activity within soil.

The fate of phenol and its derivatives in plant, such as 4-chloro-2-fluorophenol (4-C-2-FP), have been studied. Nuclear magnetic resonance (NMR) was used for the identification and quantification of pollutants' metabolites in plant extracts (Tront & Saunders, 2007). It was found that the presence of high concentration of phenol in Mango fruit has a significant influence on the anti-oxidant metabolism in 'Ataulfo' mango fruit during ripening. The result was provided by using high-performance liquid chromatography coupled to mass spectrometry techniques to analyse the individual identification and antioxidant contribution of phenol (Palafox-Carlos *et al*., 2012).

2,2-(4,4′-dihydroxydiphenyl) (BPA) is produced in massive quantities and is used in the manufacture of food cans, medical devices such as dental sealants /composites, tableware, and reusable items such as baby bottles. Study of BPA concentrations in different environmental media (aquatic, soil, sediment and air) in China, showed a higher concentration of BPA at BPA manufacturing areas. The most dangerous levels of BPA contamination was reported in southern Taiwan, where nearly 16.2 µg/L was recorded in water bodies, and 10.5 µg/g in river sediments. In addition, the study also reported the presence of high concentration of BPA in drinking water. Here, 568 ng/L was found in bottled water and 1700 ng/L BPA in the water of a baby bottle. According to the results, long-lasting effect could be expected from the extreme pollution by BPA (Huang *et al*., 2012).

Other authors also reported about phenolic compounds as being the most common soil pollutants (Delfino & Dube, 1976). Mills *et al*. (2006) reported that pentachlorophenol (PCP) has been used for treatment of wood during the last 50 years, and is the main cause of its presence in high concentration in soil and ground water. Two different sites in South Portugal were used to study the water quality and compare the concentrations of contaminants present. This research found that derivatives of phenol compounds mainly cause pollution of the water and soil. 2,4-dinitrophenol (2,4-DNP) was the main derivatives of phenol identified (Jing *et al*., 2011). This was true particularly in the month of February, when the farmers use pesticides, before planting their crop at that time of the year.

Pentachlorophenol (PCP) is a hazardous compound and is present in a high concentration in soil, making it a serious problem (Bhattacharya *et al*., 1996). Therefore, the chemical relationships between properties of soil with pentachlorophenol (PCP) was investigated (He *et al*., 2007). The degradation of some phenolic compounds has been addressed by using a Fenton system as advanced oxidation processes. The study analysed how the presence of chlorides and sulphates effect degradation of different phenolic compounds such as phenol, 2-nitrophenol and 2-chlorophenol (Siedlecka & Stepnowski, 2005).

1.6 Using Bioremediation to treat soil pollution

Bioremediation is the process of removing or neutralizing pollutants or waste from soil or water using biological agents such as microorganisms or green plants. There have been many biotechnological advancements in this technology which use the chemistry of living organisms to develop alternative technology to remove pollutants and treat hazardous waste in a more natural and environmentally friendly way (Mani & Kumar, 2014). This is a new biotechnology used for detoxification of polluted soil and water bodies, in the clean-up soil of contamination caused by petroleum hydrocarbons, industrial products, phenolic compounds and pesticides. The process depends on metabolism by microorganism such as bacteria, fungi or plants, thus making environmentally friendly and less toxic products (Donlon & Bauder, 2006; Beskoski *et al*., 2012; Calvo *et al*., 2009; Boopathy, 2000). Bioremediation has been effectively used in the treatment of soils heavily polluted by heavy metal. Using both microorganisms and plants in combination ensures a more efficient clean-up of soil that has heavy metal contamination (Chibuki & Obiora, 2014).

Bioremediation has more advantages compared to conventional techniques of removal of contaminants, such as dredging (physical removal of the contaminated sediment layers), capping (covering the contaminated sediment surface with clean material, thus isolating the sediments) and incineration (waste treatment that involves the combustion of organic substances in waste materials). Bioremediation is seen as a cleaner and cheaper method of removal of toxic contamination and seen as a non-invasive technique that does not introduce drastic changes to the ecosystem (Hoff, 1993). The earliest bioremediation technique was applied in the removal of marine oil spill in the *Exxon Valdez* oil spill in 1989 in United States. Thus, it plays a dominant role in the removal of environmental pollutants (Hoff, 1993).

Overall, the technologies of bioremediation can be classified in two main groups: *ex situ* or *in situ*. The *ex situ* technology are those that require the physical removal of the contaminated material for treatment process in other site. By contrast, the *in situ* bioremediation are these that involve treating the contaminated material at its original place of accumulation (Boopathy, 2000). For example, *Biostimulation* involves stimulating indigenous microbial populations in ground water and soil by providing essential nutrients. While *Bioventing* aims to draw oxygen through the contaminated soils in order to promote the aerobic activity of microbes (Zouboulis and Moussas, 2011).

Modern genomic tools are also being employed to investigate the systems biology of the microbial community. DNA, RNA and proteins can be analysed and associated in order to investigate how bioremediation functions at a cellular level, a community level and finally in the ecosystem (Chakraborthy *et al.*, 2012). Promising results have been reported. Wang *et al*. (2011) reported that a single strain of *Pseudomonas aeruginosa* bacteria was able to reduce phenol concentration in soil, thus promoting the growth of corn. It is suggested that the removal or reduction of phenol concentration in soil reduced the phytotoxicity of the phenol, allowing better growth of corn in the study. This is a promising prospect for recovery of contaminated land for use in agriculture.

In situ microbial metabolism of aromatic-hydrocarbon in environmental pollutants has been studied to understand how the elimination of the pollutant occurs (Jeon & Madsen, 2013).

In recent years, researchers have developed the use of microbial cells capable of degrading aromatic toxic compounds as bioremediation tools in soil, ground water and bioreactors (Gonzalez *et al*., 2001; Razo-Flores *et al*., 2003). Many attempts have been made to isolate microorganisms capable of degrading phenolic and other aromatic hydrocarbons among the Eubacteria, representatives of families *Pseudomonadaceae* (Banerjee *et al*., 2001; Mrozik & Labuzek, 2002; De Jonge *et al*., 1991).

Many authors have reported on the degradation of aromatic compounds by soil microorganism for example, *Pseudomonas aeruginosa* and *Trichoporon* (Golovleva *et al*., 1991; Banerjee *et al*., 2001). Hashem (1995;1996), isolated several isolates from soil contaminated by petroleum from Yanbu city and Al-Khafji area (Eastern region of Saudi Arabia) which belong to the following genera (*Aspergillus*, *Pencillium*, *Pseudomonas*, *Micrococcus* and *Staphylococcus*) and it was found that these genera can degrade the different petroleum compounds.

Saber & Crawford (1985) isolated *Flavobacterium* strains that which have the ability to grow and use pentachlorophenol as a sole carbon source at a concentration 100-200 ppm. The ability of some aerobic bacteria isolated from soil to degrade 2, 4, 6- trichlorophenol contaminated soil under special laboratory conditions was also investigated (Sanchez *et al*., 2004). They can degrade about 50% of this compound after incubation for 30 days.

Bioremediation was applied to remove polynitroaromatic compounds such as TNT from the soil. Although 2, 4, 6-trinitrotoluene (TNT) degrades very slowly, it can be used as nitrogen source by a number of microorganisms (Peres, 2000).

Despite having discovered an extensive range of microbes capable of degrading numerous toxic organic compounds, the environmental contamination by these compounds is still unsolved. More work is needed to be done (Azaizeh *et al*., 2011).

1.7 Phytoremediation

The technology of using green plants to remove contaminations from the soil or groundwater or to make them non-toxic is called phytoremediation (Kochian, 2000; Macek *et al*., 2007). Phytoremediation using combined action of plants and microorganism in the rhizosphere is promising too (Harvey *et al*., 2002).

This can involve removal, transformation or immobilization of toxic compounds located in soils, sediments or water (Truu *et al*., 2015). Phytoremediation can be used to treat contaminants such as petroleum hydrocarbons, chlorinated solvents, pesticides, explosives, heavy metals and radionuclides (Truu *et al*., 2015; Macek *et al*., 2007). This approach is said have many advantages compared to conventional methods. This technique is cheaper, less disruptive to the environment, has better public acceptance and has potential to treat multiple pollutants at a time (Truu *et al*., 2015).

It is one of several types of bioremediation. In some plants, their capability is exploited to uptake contaminants from the soil to sort them in appropriate part of the plant such as shoot or roots (Hinchman *et al*., 1996). However, phytoremediation technology has different approaches that can be applied for the remediation of polluted soils and water (Hooda, 2007). For example, Rhizofiltration; using roots to clean up the contaminants from water and soil. Phytoextraction; aimed at the removal of contaminants from soil by using plants and

accumulation in plant tissue. Phytovolatilization; involves the absorb of contaminants from soil or water and volatilize them subsequently by plant transpiration into the atmosphere (Campos *et al*., 2008).

Bioremediation and Phytoremediation are environmental technologies that use microbes and plants respectively to clean up the organic contaminants from the soil (Kang, 2014; Schwitzguebel *et al*., 2011). Phytodegradation process to clean up the environment of the organic compounds, including petroleum and aromatic compounds in soil, volatile compounds in air or solvents in groundwater, can occur inside the plant or within the rhizosphere. Thus, it is essential that more studies in this field are carried out (Newman & Reynolds, 2004).

In phytoremediation, it has been found that plant roots play and important role in the removal of organic pollutants (De Araujo *et al*., 2002). To sum up, using plants for degradation, metabolism and decontamination can remove many organic chemical contaminations (Susarla *et al*., 2002). For the metabolism of plants, phenol is presented in a high-amount often in the environment. Some researchers have investigated the degradation of phenolic compounds by plants. For example, the relation between removal, uptake, accumulation, and toxicity of phenol in willow trees was studied (Ucisik and & Trapp, 2006).

In plant tissues, organic contaminants are usually present in derivative forms as well as parent compound. For example, they can be found covalently bound to polymers in the walls of cells in plants (Tront & Saunders, 2007). Ugrekhelidze *et al*. (1999) carried out a procedure with aseptic mung bean and wheat seedlings to determine the pathways of phenol decontamination. The transformation of ^{14}C phenol during the penetration of plant's roots was investigated. It was found that phenol conjugates with low- molecular-weight peptides. Those contribute to the formation of a coupled phenol-peptide. The research assumed that the hydroxyl group of phenol and the functional groups of peptides played a role to achieve the conjugation. It is suggested that the main pathway for detoxification of phenol is likely to be caused by this conjugation with low-molecular-weight peptides. Aromatic ring cleavage and a dibasic carbonic acid formation lead to the metabolism of a small proportion of the phenol as it penetrates through the roots. On the other hand, the molecular penetration of phenol contributes to enhance the presence of peptide synthesis in the plant's cell. The plants take up approximately 80 % of the applied phenol (Ugrekhelidze *et al*., 1983).

Phytoremediation has some benefits such as being a novel technique, efficient and cheaper than other methods like physical, chemical and conventional methods (evaporation and chemical

reduction). There is a need to have more knowledge to understand the complicated interaction between plant and rhizosphere that cleans up the contaminants from the soil (Hooda, 2007; Chinmayee *et al*., 2012). Phenolic compounds pose a risk to the environment and human health even at a low level of concentration. Consequently, using phenol in the context of phytoremediation has been investigated. Vetch, which is a species of legume, was tested. The plant was successful in removing phenolic compounds from the soil (Ibanez *et al*., 2012).

Phytoremediation of excess phosphorus and nitrogen was successfully achieved using *Canna x generalis* from secondary waste water. However, it was not able to remove phenolic compounds (Ojoawo *et al*., 2015). Phytoremediation was successfully tried in Egypt, using *Plantago major* L for removal of an insecticide called cyanophos. Cyanophos is commonly used in Egypt to control various agricultural and horticultural pests. It is highly persistent and accumulates in rivers and lakes (Romeh, 2014). *Plantago major* L shows a great potential as a useful plant for pesticide phytoremediation. Phytoremediation of solvents is said to be almost at the point of being considered as an accepted technology (Newman and Reynolds, 2004).

There was a study, which demonstrating that willow (*Salix* sp., 'Tangoio') and poplar (*Populus* sp. 'Kawa') can survive with up to 250 mg kg^{-1} of PCP in soil pollutants. The breakdown of PCP in both pots with plant and without plant was determined. It was more in the pot with plants compared to the other. In the application of phytoremediation using an irrigation system, data showed that there was a decrease in soil hydraulic conductivity when they reused the technique of leachate containing PCP and metal compounds (Mills *et al*., 2006). It was found that the degradation of 2,4-dinitrophenol depended on a variety of factors such as pH, the type of soil and season. 2,4-dinitrophenol in soils was adsorbed easily when there was a high clay content with low level of pH. These conditions delay the process of biodegradation and leaching of 2,4-dinitrophenol. The study also showed that, the only site where the concentration of 2,4-dinitrophenol was very low was where high vegetation was found. This demonstrates that plants play a dominant role in cleaning up the contaminants (Barrico *et al*., 2006) and will lead us to the objective of this current study.

It is important to acquire knowledge in identifying specific pathways and possible proteins involved in the biodegradation process in order to further improve and exploit it for human benefit. In recent years, significant biotechnological advances had been made in phytoremediation by using tissue culture and genetic engineering techniques to modify plants for metal uptake, transport sequestration (Eapen & D'Souza, 2005). Oller *et al.* (2005) reported

that over-expression of basic peroxidase in transgenic tomato (*Lycopersicon esculentum*) hairy roots increased phytoremediation of phenol. Tomato (*Lycopersicon esculentum*) was found to have high levels of peroxidases that are capable of removing phenols. The gene named *tpx I* encodes a basic peroxidase in tomato roots. Oller *et al*. (2005) overexpressed this gene and improved the efficiency of phenol removal by the plant.

Gene expression studies are also used in metal phytoremediation processes. Over 450 plant species are reported to be characterised as metal hyperaccumulators (Halimaa *et al*., 2014). These plants could possible help extract metal from contaminated or metal-rich soils. Specific genes have been associated with the ability to accumulate a certain metal by a plant. Halimaa *et al*. (2014) studied gene expression differences between *Noccaea caerulescens* ecotypes in order to identify candidate genes for metal phytoremediation. The study showed that *Noccaea caerulescens* has evolved a great diversity in expression of metal-related genes, making it able to adapt to various metalliferous soils. Using this type of information we can develop plants with improved ability to accumulate and tolerate metal, thus develop improved plants for metal phytoremediation.

1.8 Integrating Plant, Microbes etc.

In attempts to discover how the microorganisms, plant roots and soil interact, the physical and chemical of soil properties have been extensively investigated (Hinsinger *et al*. 2005).

To shed light on how the roots effect the physical and chemical soil properties, several methodologies and models have been used. For example, how the exudation of microorganism interacts with root and soil. Secondly, using a microcosm technique to study the impact of root on different soil. Finally, the rhizosphere soil was sampled and characterized at various distances from plant root (Luster *et al*., 2009).

The interaction between plant roots and microbes has been investigated, in order to understanding the microbe's role in plant health. The study demonstrates that plants are capable of choosing and governing the rhizosphere microbiome. Also the study illuminated how the plant exploits the microbe's activity in order to protect it from insect attack. This information supports the need for knowledge in order to realize the dominant role of plant roots in mechanisms to control and choice of microorganisms and its activity for increased crop production (Berendsen *et al*., 2012).

Therefore, microbes, plants and plants-microbes together were used (Ramos *et al*., 2005). As roted above, in the context of microbial biodegradation, a number of microorganisms have been reported with capability to degrade phenol and other phenolic compounds at a low concentration.

In Saudi Arabia, with the establishment of industrial cities such as Yanbu, there are many efforts to save the environment from pollution. For example, plant species growing in the industrial area have been studied due to the important role of interaction between leaf- and rhizosphere-associated bacteria to remove contamination of environment. In addition, the study identified some strains of bacteria isolated from leaves and rhizosphere that were capable of removal some contaminants such as phenol in the soil and water (Khiyami, 2008). El-Kateeb *et al*. (2014) reported the isolation of two species of bacteria; *Bacillus sp* and *Rhodococcus sp* and two species of fungus; *Candida tropicalis* and *Phanerochaete chrysosporium* from olive mill waste with the ability to degrade phenols. This waste accumulates high phenols in the environment. It is important that the mode by which the reduction of phenol is achieved by the microorganisms is studied and further improved.

In addition, a soil microcosm has been used to evaluate the impact of remediation under artificial conditions, of diesel fuel in soil, during a 180-day experiment, using native soil microflora, earthworms and plants in an attempt to study the interaction between them. The study revealed that the decline rate of diesel in soil was due to earthworms, while the grass played a role in stimulating microbial biomass. The research showed that phytoremediation had no effect on hydrocarbons degradation in the short term compared with the effect of earthworms (Fernandez *et al*., 2011). In addition, Mikolasch *et al*., (2015) isolated several strains of microorganism from the rhizosphere of plants that were grown in soil contaminated with crude oil such as alfalfa (*Medicago sativa*). Three of these strains was selected to inoculate with barley seeds and sown in oil-containing sand to study the influence of microbes on the growth of plant. These treatment help to improve the growth of barley seedling in oil-containing sand compared to uninoculation plants. This finding may be useful to apply in rhizoremediation projects.

1.9 Experimental approach

1.9.1 Using microcosm system to study root-soil interaction

The aim of this project was to study the effect of plants on the removal of phenolics as contaminants. The rhizosphere is the region of soil around living roots, which is influenced by root activity and vice versa (Hinsinger *et al*., 2009; Neumann *et al*., 2009). Using a combination of phytoremediation and microbial bioremediation strategies, a new and more successful approach to remediate contaminants called rhizoremediation or bacterially-assisted phytoremediation is being developed (Glick, 2010).

In the natural environment, soil obscures root system visualization *in situ*. Roots can form extensive networks in the soil. This trait prevents their easy extraction for observation. Therefore, complementary laboratory and greenhouse approaches have been devised to overcome these limitations. For example, to better access the root system, plants can be grown in mesocosms of soil (Zhu *et al*., 2011). While mesocosms may be able to reproduce conditions closer to the field environment, visualization of the root system remains a challenge (Zhu *et al*., 2011). According to Neumann *et al*. (2009), some of the major challenges of rhizosphere research in plant sciences include detection and quantification of root distribution under natural soil conditions and monitoring of root activity *in situ*. This requires related methodological approaches, including tools for monitoring root growth, for rhizosphere sampling, and the detection and quantification of root activity with respect to nutrient uptake in the rhizosphere. In addition, tools are essential for visualization and quantitation of root growth to investigate specific root details in soil (Metzner *et al*., 2015). X-ray Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) are suitable for such studies. Both techniques achieved equally well with small diameters suited to monitor root development of bean seedlings. Although CT was valuable in providing the higher spatial resolution, the corresponding information can be obtained using a combination of the two techniques and could open a whole range of further possibilities, like the analysis of root system behaviours in different soil structures or under varying soil moisture (Metzner *et al*., 2015).

Recently, the microcosm technique has been applied in different sizes in context of studying environmental toxicology. There are many benefits to take into account such as cost, scale, making it easier to carry and control conditions with more realistic results than natural field

environment (Chen & Edwards 2001). Consequently, the microcosm system was used in this project to study the effect of plant in phenolic compounds removal.

1.9.2 Sampling approach of soil solution

The use of microcosms goes a long way to solve the problem of accessing the roots in the rhizosphere. This technique can then be used to tackle the pressing question of what is happening at root/soil interface. Moreover, in this project we move to a far finer scale of measurement using modification of the SiCSA approach (Tandy *et al*., 2013). Valuable information was gained.

According to Tandy *et al*. (2013), the plant / soil interface is important as a distinct micro-site for physicochemical and biological processes. These correlated processes are complex and need to be understood over a wide range of spatial and temporal scales. The processes averaged by large sample scales may lead to the loss of vital resolution. Single Cell Sampling and Analysis (SiCSA) is a battery of micro-analytical techniques developed (Tomos & Leigh, 1999; Tomos & Sharrock, 2001) to measure the physical and chemical properties of individual cell within complex tissue. At Bangor University, the principle has been applied to soil-most successfully by Tandy *et al*. (2013).

1.9.3 Analysis of soil and plant samples

The SiCSA technique allows obtaining small samples (within the range of pl to nl). This small volume requires very sensitive analytical technique. Capillary Zone Electrophoresis (CZE) can be used for the analysis of small molecules and ions from the plant cells and heterogeneity of soil surrounding the root at very high resolution. A glass microcapillary allows small samples to be obtained and manipulated under paraffin oil before being introduced to CZE for the analysis (Bazzanella *et al*., 1998).

Capillary electrophoresis has been used for molecular separation with a wide application, including the analysis of different molecules sizes and ionic species (Kremser & Kenndler, 2004). In the last few years, capillary electrophoresis is considered to be one of the most powerful techniques for phytochemical analysis. Crucially in this current work, only a low injection volumes is required (in the nanoliter-range for 75 and 50 µm capillaries). This opened

the possibility for analysis at a single cell level (Bazzanella et *al*., 1998) or at sub mm scale in the current work.

Using it, Tandy *et al*. (2013) investigated the interaction between (depleted) uranium (DU) and exudates of root, in order to demonstrate the fate of DU. A modified version of the SiCSA approach was used to sample the soil solution and Capillary Zone Electrophoresis (CZE) was used for the sample analysis.

Recently, CZE was applied to the analysis of phenolic compounds due to its superior efficiency in separation (Tsai & Her, 1996; Lima *et al*. 2007). The optimization of phenolic compound analysis using CZE was done using tetraborate buffer in order to find a simple and rapid analysis method (Lima *et al*., 2007). Consequently, in this study, phenolic compounds were analysed in the soil and plant using a CZE technique.

1.10 Conclusion

In conclusion, contamination of soil is a growing problem. There is a need to better understand how plants can deal with contaminating compounds especially phenol compounds. They are of great importance for the environment not only due to this toxicity but their ability to resist natural biodegradation process. Thus, they accumulate in high concentration in the natural environment.

Phenol is a pollutant that affects many regions in the Kingdom of Saudi Arabia. As one of the major petroleum producer in the world, there are many oil wells, petroleum refineries and petroleum related manufacturing work that pollute the environment with phenol. Apart from petroleum related business, agriculture based industry is also a cause for concern in phenols accumulation. In Saudi Arabia, the Al-Jouf region as one of the major olive oil producers (Amany & Maliha, (2014), the olive mill waste (OMW) can accumulate phenolics and pollute the agriculture land in the surrounding region. Phytoremediation would be an ideal solution for this issue. In this program, a unique and totally novel assembly of techniques was used to achieve this.

1.11 Objectives

The aim of this study was to look for the potential of using plants to clean up soil polluted by phenolic compounds (phytoremediation). This included several subsidiary objectives.

Firstly, the study sought to assess the influence of microbes on the removal of phenolic compounds by measuring the behaviour of these compounds in non-autoclaved soil and autoclaved soil (control) (Chapter 3).

The second objective was to stimulate *ex planta* effects on these compounds using a microcosm system. Two plant species were chosen, rye and wheat, to assess the influence of plant presence on the removal of phenolic compounds. Both low and high resolution mapping were used. In addition to examining the general e*x planta* effect on phenol disappearance from the soil, further work looked at whether any effect was due to direct secretion of enzymes or by the plant inducing microbes to secrete the enzyme (plant-microbes association) (Chapter 3, 5 and 6).

Finally, the study then sought to investigate the direct uptake of phenol and one of its derivatives (syringic acid) by a wheat root and to compare the pathways of these compounds in the plant using low and high resolution mapping (Chapter 7).

An additional chapter discusses technical difficulties that arose during the fine scale analysis of phenol distribution (Chapter 4).

Chapter 2: Material and methods

2.1 Soil and plant material

Uncontaminated loam, silty loam soil (Sannan Series) (Ball, 1963) was collected from Henfaes Farm, Bangor University, North Wales, UK (53˚ 14'N, 4˚ 0'W) and used for this study. Soil (50 kg) was stored in unsealed plastic sacks in the dark at room temperature and used over a period of one year. In some experiments, only the soil was used (Chapter 3, section 3.2) while in all the other experiments soil + sand were used to facilitate the sampling of soil (see section 2.6.1).

The wheat seeds (Variety Hereward) were harvested in the 2007 Season at Bishops Farm, Oldberrow, Henley-in-Arden,Worcs (provided by Dr K.A. Steele). Variety Hereward seeds (2010) were supplied by OpenfieldTM, (www.openfield.co.uk). Organic rye seeds were supplied by Healthy Supplies Ltd, Brighton, (www.healthysupplies.co.uk).

2.2 Soil moisture content

To measure the moisture content of the soil, approximately 10 g of the soil (in triplicate) was weighed, dried in open petri dishes at 50° C and then reweighed after 24 and 48 hours. No further weight loss was observed between 24 and 48 hours. The 24 hours value was used as the estimate of dry weight. Moisture content was calculated from equation (2.1):

$$M\ (\%) = \frac{W - D}{W} \times 100\ \% \qquad \textit{Equation}\ (2.1)$$

(Where M= moisture content, W= wet weight and D= dry weight).

2.3 Soil Autoclaving

A 250 ml Duran glass bottle was used to autoclave 100 g samples of soil. The soil was autoclaved at 121ºC for 30 minutes at15 psi before the addition of phenolic compounds.

2.4 Measuring the disappearance of phenolic compounds from extractable moisture in non-autoclaved and autoclaved soil

Eight phenolic compounds were selected to determine their disappearance from the soil (Table 2.1) phenol, 3-chlorophenol , 2-chlorophenol , 2,4-dinitrophenol, were chosen as representative contamination compounds, while the other four phenolics are biological compounds which are present in lignin and humic substances (Lima *et al*.,2007): 3, 5 dimethoxy-4-hydroxyacetophenone, ethylvanillin, vanillic acid and syringic acid. Each compound (10 ml) was mixed separately with 100 g of non-autoclaved and autoclaved soil. The moisture content of soil before the addition of the phenolics was 7% (section 2.2). To determine the concentration of phenolic compounds the soil was extracted using centrifugation (section 2.7.1) and the extract analysed using CZE (section 2.10).

Compounds Used	Concentration (mM)	Boiling point	Polarity
Phenol [A]	20	181 °C	Less polar
3-Chlorophenol (3CP) [A]	20	214 °C	Less polar
2-Chlorophenol (2CP) [A]	20	175 °C	Less polar
2,4-Dinitrophenol (2,4DNP) [A]	2	113 °C	Less polar
3, 5-Dimethoxy-4-hydroxyacetophenone (DAHMP) [B]	10	335 °C	Less polar
Ethylvanillin [B]	10	295 °C	Less polar
Syringic acid [B]	20	380 °C	Polar
Vanillic acid [B]	2	353 °C	Polar

Table 2.1 Eight phenolic compounds were selected to determine their degradation into soil: four are artificial [A] and four are biological compounds [B].

2.5 Microcosm system

A microcosm (Figures 2.1, 2.2 and plate 2.1) was made up using acrylic sheet (supplied by PlasticSheets.com, Leicester, UK) with three ridged sides with dimension of 16 × 8 cm to hold 3 mm thickness of soil. It was then covered with three pieces of glass (7.5×5 cm) microscope slides (Supplied by XYZ Microsupplies Ltd) to overlay the soil inside the microcosm. These glass pieces divide the microcosm into three equal sized parts (top, middle, and bottom; Figure 2.1). An acrylic sheet measuring (16 × 8 cm) formed a lid on top of the three glass microscope slides to seal the microcosm (Figure 2.1). It was covered tightly using a piece of black plastic

to avoid phototropic effect and algal growth and held firmly in place using two clips. The cross section of the microcosm would appear as below (Figure 2.2 and plate 2.1).

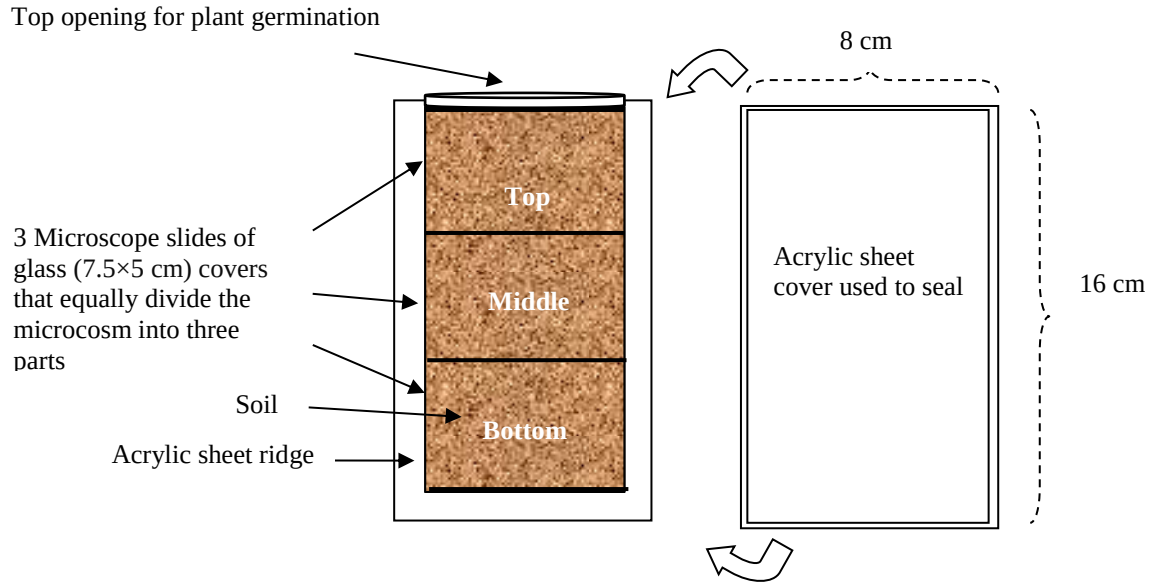


Figure 2.1 Schematic diagram of microcosm, showing acrylic sheet with three microscope slides. Each slide measured 5 cm by 7.5 cm length by width.

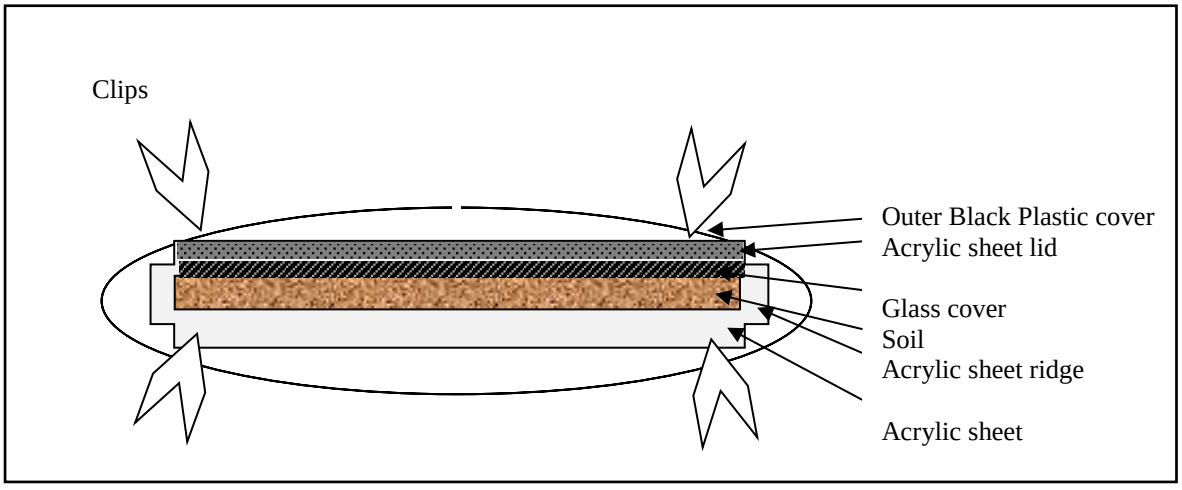


Figure 2.2 Cross section of the microcosm.

Plate 2.1 Microcosm (see Figures 2.1 and 2) filled with 50 g of soil and covered with black plastic.

2.6.1 Plant growth

Plant seeds were first soaked in 100 ml of tap water in a conical flask for 24 hours. During this time the seeds were submerged under water and the flask was aerated using an air pump. Three germinated seeds were chosen to sow in each microcosm.

Soil (375 g) was mixed with 125 g of sand and 125 ml of water. After mixing, 50 g of that was used for each microcosm. Three plant seeds (section 2.1) were sown in each microcosm (plate 2.2). The seedlings were grown for 4 days in a microcosm in a growth room at 23°C in continuous light. Light intensity at leaf level was 480 µmol $m^{-2}s^{-1}$. In a growth chamber, microcosms were placed at an approximately 30°angle, face down to increase the root number accessible for sampling (Tandy *et al*., 2013). Each microcosm was opened and sprayed daily

with 7 ml of tap water. The moisture content of plant microcosm was 9%. (This was influenced by the uptake of water by plant or leakage of water from the bottom of the microcosm).

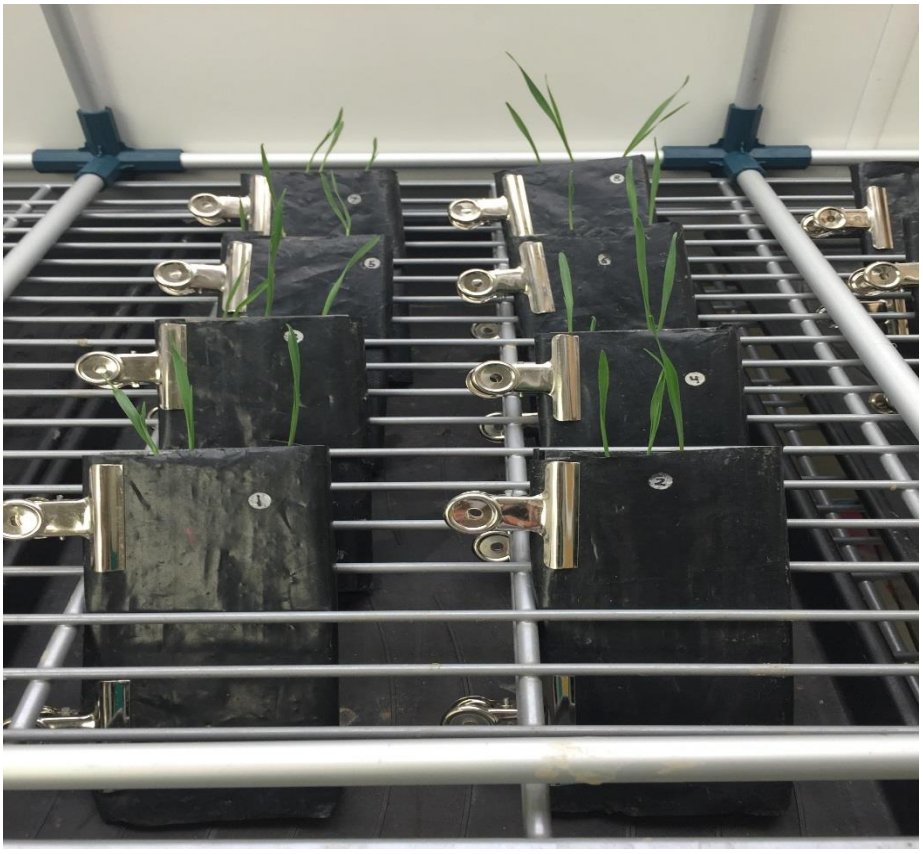


Plate 2.2 Wheat microcosms under control condition.

2.6.2 Measuring transpiration rate

The weights of three wheat microcosms (4 days old) were measured (Time 0). They were measured at 24 hours to calculate the transpiration rate. Loss of microcosm weight was considered equivalent to the water transpired in 24 hours.

2.7 Adding phenolic compounds to the microcosm

2.7.1 Using spray method

Two microcosms were used. One microcosm had soil only and the second microcosm had soil with wheat or rye seedlings. Both were sprayed with 5 ml of 20 mM of phenol (as an example of the phenolic compounds). Each one spray was equal to 1 g. First spray was in the top of microcosm, the second one in the middle, third one in the bottom and the fourth one between the top and the middle of the microcosm, and fifth spray was between the middle and the

bottom. The distance between the spray and soil microcosm was approximately 3 cm and the slides were set standing around the microcosm to avoid the loss of phenol. At time intervals, soil sample of approximately 0.5 g was taken by spatula from three different areas; top, middle and bottom (Figure 2.1) of each microcosm. A pore about 1 mm size was made using a needle at the tip of a 1.5 ml Eppendorf tube. 0.5 g soil was added into this tube. This tube was transferred into another 1.5 ml tube (Plate 2.3) and (together) centrifuged in a microfuge (Heraeus sepatech, Biofuge 15) for 5 minutes at 13,000 g. The liquid collected in lower Eppendorf tube was used for analysis. The samples were taken at 30 minutes, and at 24 hour after addition of phenol. The distribution of phenol following spraying was determined by measuring the phenol concentration in soil solution using Capillary zone electrophoresis (CZE) (see section 2.10).

2.7.2 Adding phenol from the top

In another experiment, phenol solution (5 ml of 20 mM) was mixed with 5 ml of water then added to the soil and wheat microcosms from the top. Again, soil sample was taken and analysis as described in section (2.7.1).

The purpose of this experiment was to determine the most appropriate way to introduce phenol to the microcosm by spraying or by adding it with water from the top of the microcosm. The distribution of phenol following spraying was relatively uniform (Figure 2.3) while significant differences were found between the concentration of phenol at the top compared with the middle and bottom areas when it was added with water from the top (Figure 2.4). Therefore, using the spraying methods to add the phenol was chosen as the method to use in the following experiments.

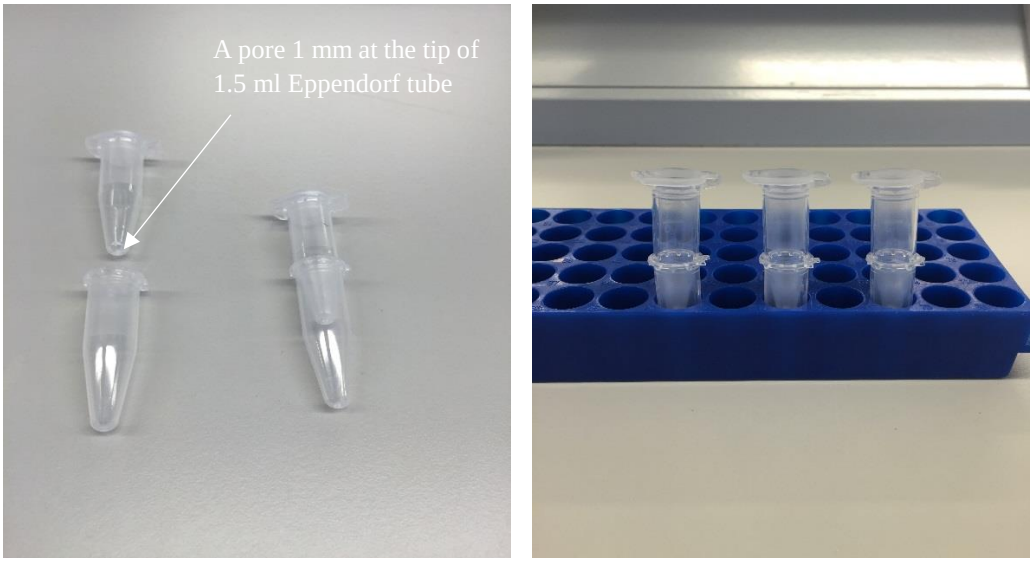


Plate 2.3 A pore about 1 mm size was made using a needle at the tip of a 1.5 ml Eppendorf tube. This tube was transferred into another 1.5 ml tube after adding of soil sample to centrifuge.

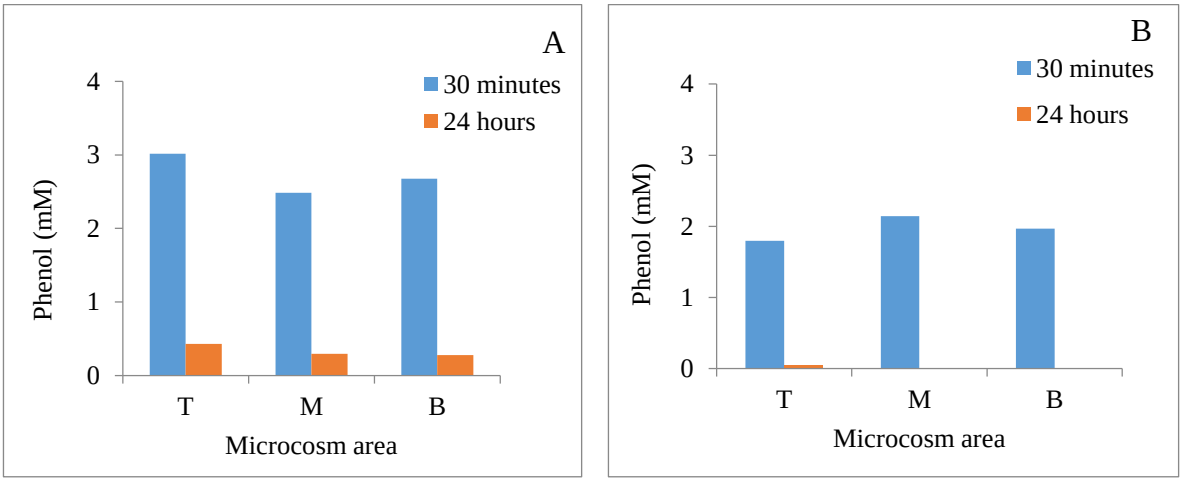


Figure 2.3 The concentration of phenol at 30 minutes and 24 hours after the addition 5ml of 20 mM phenol to the microcosm by spraying. (A) microcosm without plant (B) microcosm with plant. T = top, M = middle, B = bottom. n=1: only single sample was tested.

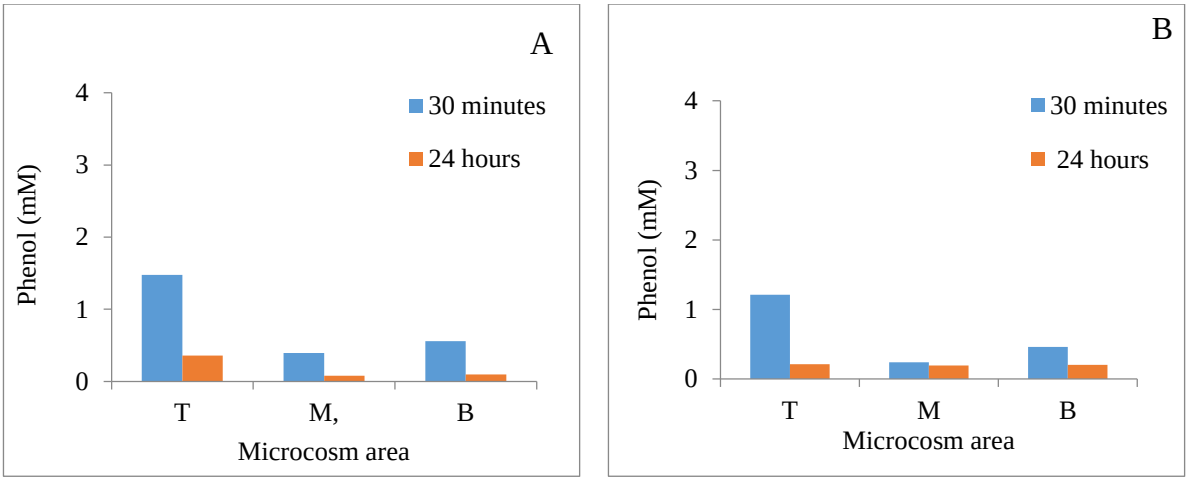


Figure 2.4 The concentration of phenol after mixing 5 ml of 20 mM of phenol with 5 ml water then adding to the microcosm from the top (A) microcosm without plant (B) microcosm with plant at 30 minutes and 24 hours. T = top, M = middle, B = bottom. n=1: only single sample was tested.

2.8 Soil solution

Other phenolic compound solutions were added onto the soil as a described above for phenol. The disappearance of these compounds from the soil in the presence and absence of wheat and rye roots was measured. To achieve this, soil samples were taken from the microcosm to determine phenolic compound concentration. Soil solution was obtained by two different methods:

2.8.1 Centrifugation (macro approach)

Soil material (approximately 0.5 g) taken from a microcosm using a spatula was centrifuged at 13,000 g for 5 min at room temperature to obtain the soil solution (section 2.7.1). Its composition was analysed using CZE at room temperature (section 2.10).

2.8.2 SiCSA Technique (micro approach)

A modification of the single cell sampling and analysis (SiCSA) technique (Tandy *et al*., 2013) was used to sample soil solutions from the microcosm rhizosphere at fine resolution (~2 nl) using a micro-capillary (plate 2.4 A & B). The sample was stored under water-saturated paraffin oil (supplied by BDH Laboratory Supplies, Poole, Dorset, BH15 1TD, England) to prevent evaporation and contamination (Tandy *et al*., 2013), before analysis using CZE (Plate 2.5). Soil was mixed with sand (3:1 ratio) to facilitate the sampling of soil by micro capillary. During the sampling, the microcosm needed to be exposed to air and this might have caused dehydration,

which might have affected the concentration of phenolic compounds measured. The boiling point of phenol is 181.7 ºC. Therefore, it may evaporate slower than water resulting in an increased apparent concentration. The rate of evaporation was measured in the next experiment.

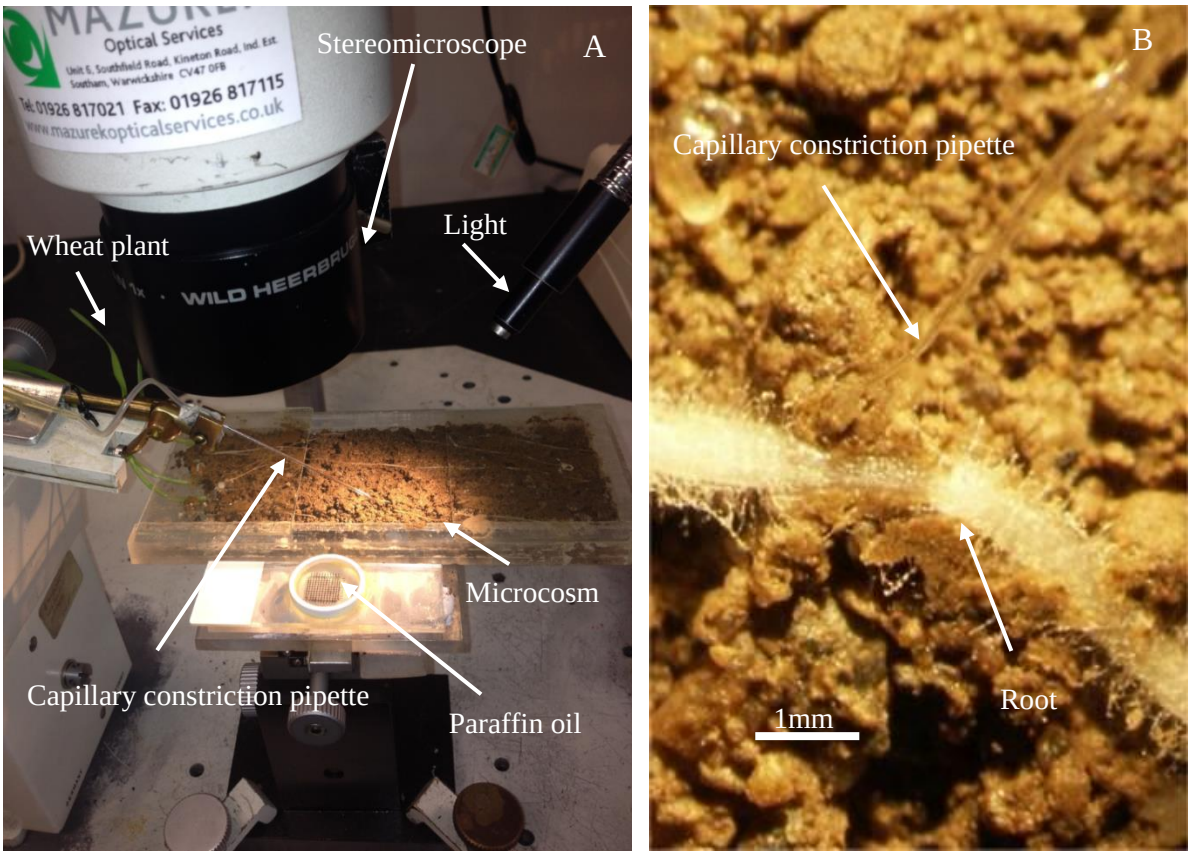


Plate 2.4 (A) Root sampling by capillary constriction pipette from small microcosm. **(B)** Example of soil sampling (from rice root, Morizuka, unpublished). A 5 nl soil solution occupies approximately 1mm^3 of soil.

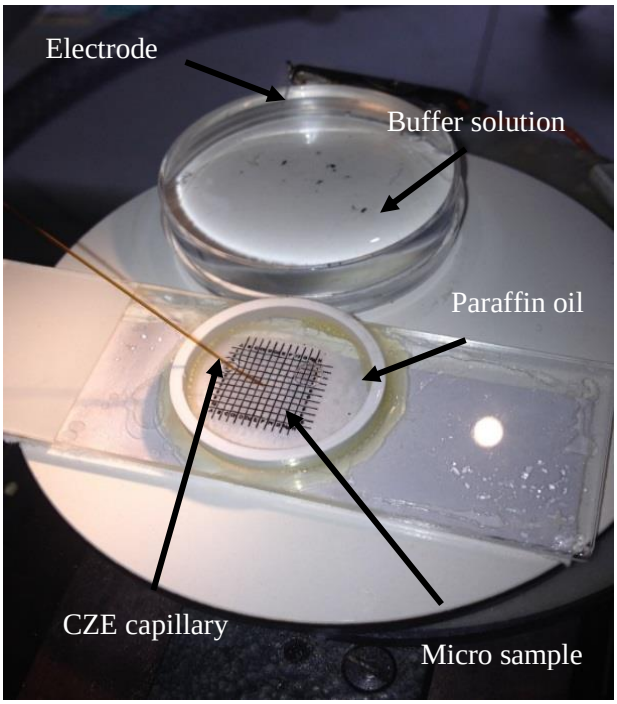


Plate 2.5 Micro sample stored under paraffin oil prior to analysis by CZE.

2.9 Measuring the rate of evaporation from microcosm during analysis

In the modified SiCSA technique (Tandy *et al.*, 2013) the lights from the microscope may have had an effect on the moisture content of the soil microcosm. Tandy *et al.* (2013) used moistened filter paper to surround the window of the microcosm to limit the drying of the soil during the process of sampling. An experiment was performed to estimate the amount of evaporation of water from the microcosm as this might have had a bearing on the measured phenolic compounds' concentration in the soil.

The weights of three microcosms were measured on an electronic balance every minute over a 20 minute period under the illumination of fibre-optic cold light (Intralux5000, Volpi). The distance between the soil and the light was approximately 4 cm. This is the same condition as that used during micro sampling experiments (Plate 2.4.A). As a non-illuminated control, the weight of another three microcosms was estimated away from the light during the same period at room temperature (23 ˚C).

It was found that in the presence of the light the weight of (50 g soil) microcosm reduced by 9 mg/min ± SE (n=3) to the end of the experiment (Figure 2.5 A). While the fall in the control microcosms weights was similar to the decrease of weight under microscope light at 11 mg/min

± SE (n=3) (Figure 2.5 B). Consequently, the percentage of water evaporation after 20 minutes in both conditions was very low (0.006% and 0.008% respectively and not significantly different from each other (P = 0. 090, Sig (2-tailed)). The precautions used by Tandy *et al.* (2013) would appear not to the necessary over an experiment period of several hours used here. However, if the experiment needed to be run for several days this effect would become important and a method of reducing or replacing water loss would need to be devised.

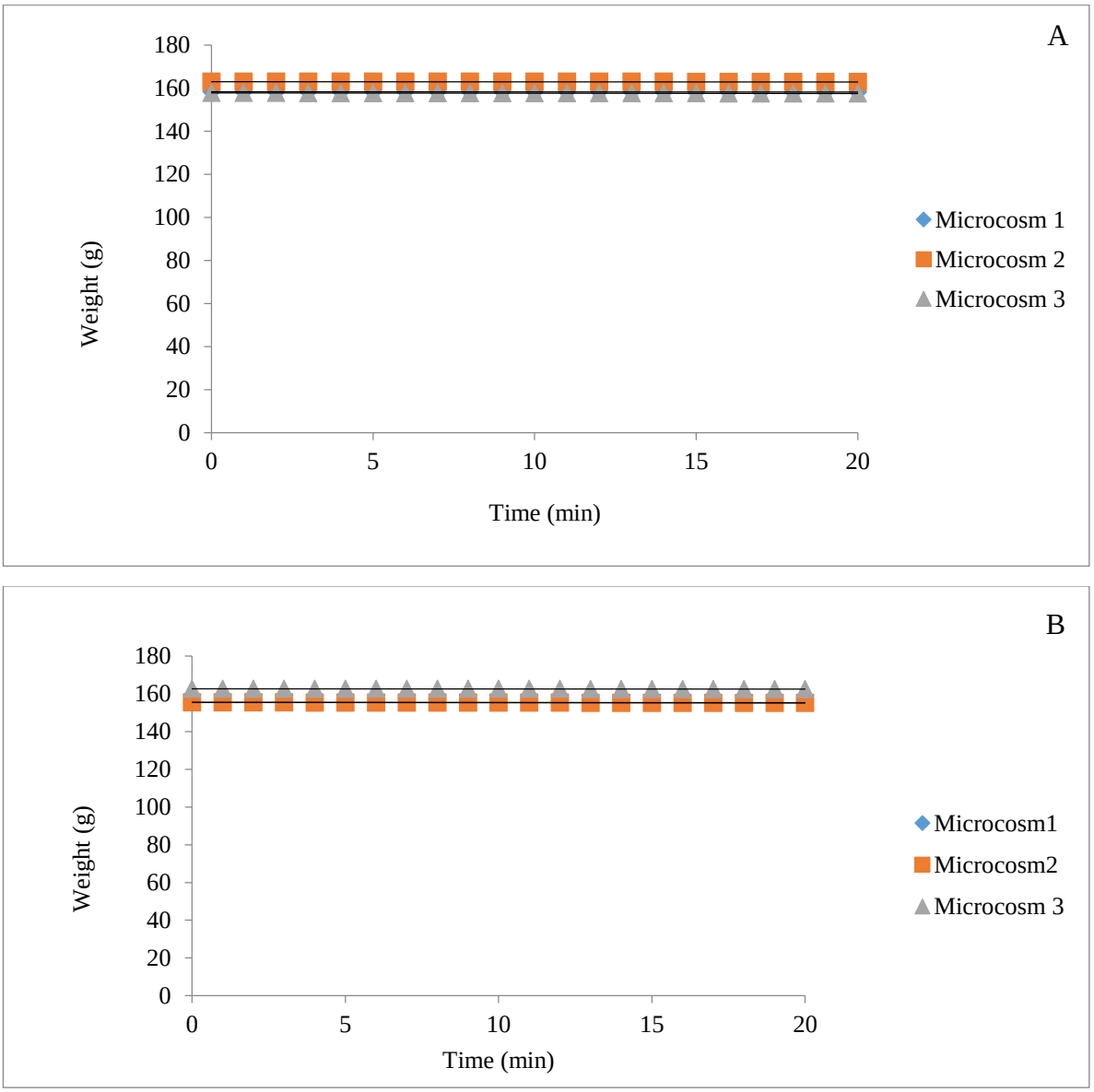


Figure 2.5 The reduction of microcosm weight due to water evaporation during analysis. (A) Under microscope light. (B) In the absence of light (control).

2.10 Capillary zone electrophoresis (CZE)

Capillary zone electrophoresis was used to determine the concentration of phenolic compounds (Plate 2.6). A custom built CZE, equipped with a Lambda 1000 UV detector (Bischoff, Leonberg, Germany) and a high voltage power supply (HCN 6M 30000, Omiran Ltd,Suffolk) (Tandy *et al*., 2013) was used. Capillaries of fused-silica, with 50 µm internal and 365 µm external diameter (Composite Metal Services Ltd, W, Yorks, UK) (60-80 cm long) were used (Tandy *et al*., 2013). Electrophoretic separation was achieved at the negative power supply of -20 kV and a current of ∽50 µA. Solutes were monitored by direct UV absorption at 240 nm. Sample was added to an equal amount of the internal standard (see the results sections for choices of standard). A sodium tetraborate buffer was used contained 43 mM $Na_2B_4O_7$, 27 mM KH_2PO_4 and 8.5% CH_3CN solution with a final pH of 9.3 (Lima *et al.*, 2007). The software used for data processing was Clarity Lite Chromatography version 5.0.3.185, (DataApex Ltd). Figure (2.6) shows an example of phenolic compounds separated in tetraborate buffer.

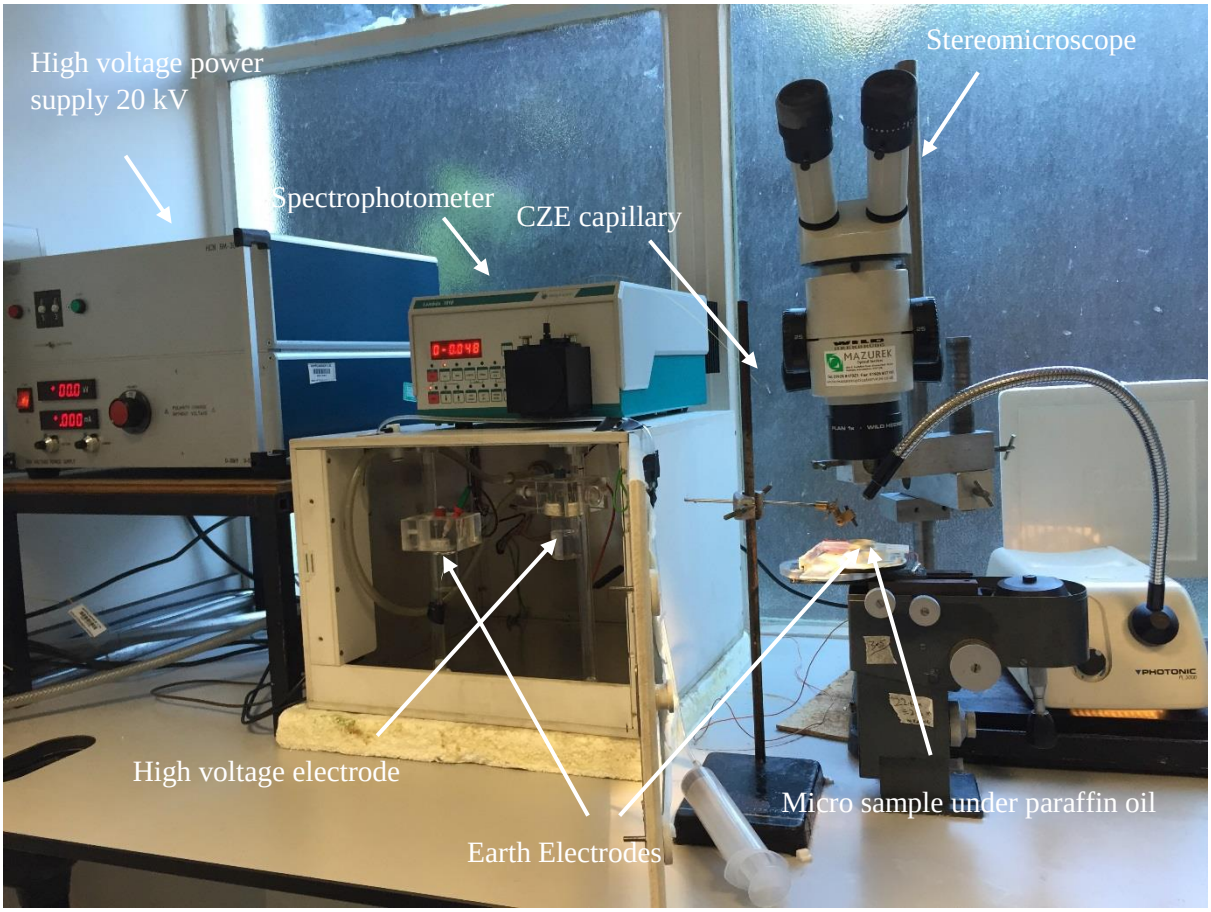


Plate 2.6 CZE Experiment setup for the analysis of macro and micro samples from soil solution or plant tissue extraction.

All the chemicals were purchased from Sigma Aldrich. Purified water (18 M Ω cm^{-1}) Elga UHQ was used for the preparation of all solutions. It is both (RO, DI) and bacteria filtered.

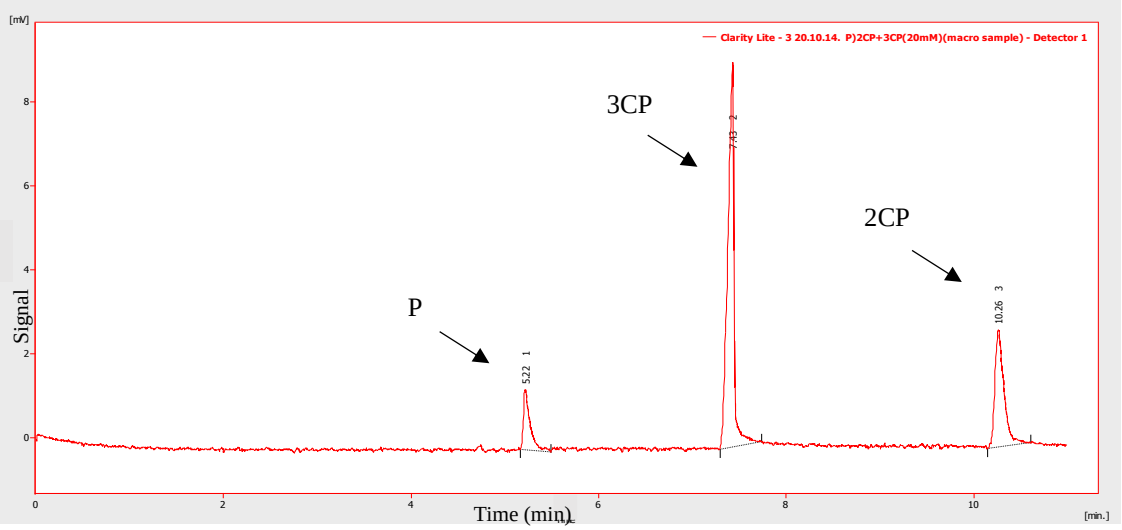


Figure 2.6 Phenolic compounds separated in tetraborate buffer (P, phenol), (2CP, 2Chlorophenol) and (3CP, 3Chlorophenol). (CZE run as described in section 2.10).

In the macro approach, a siphon injection was used to inject of the sample into the analytical system. A siphon was set up by replacing the earthed electrode buffer (inside the box, Plate 2. 6) with the sample solution and lifting this 5 cm relative to the power electrode buffer. After a one minute time interval this solution was lowered to that of the power electrode level and then replaced by buffer before applying the running voltage.

2.11 pH of the soil

pH of autoclaved soil (at 121ºC for 30 minutes at 15 psi) and non-autoclaved soil was measured using a modified version of Tandy *et al*. (2013) methods. Soil (10 g) was mixed with 25 ml deionized water in 50 ml falcon tube. After shaking, this was allowed to settle and the pH of the supernatant was measured using an Electronic Instruments Ltd, pH Meter 7020.

2.12 Leaf and root extraction

2.12.1 Phenol

Plant tissue was rinsed with purified water (18 M Ω. cm^{-1}; Elga UHQ) then placed in a 1.5 ml Eppendorf tube. A hole was made in the tube tip and cell sap obtained as describe in section

(2.7.1) following freezing in liquid nitrogen and thawing (Fricke *et al*., 1994). Samples were mixed with equal amount of internal standard before analysis using CZE (section 2.10).

2.12.2 Syringic acid

A glass microcapillary was used to extract individual cell sap directly from root (10 mm from root tip) (Pritchard *et al*., 1996) and leaf cells of wheat to quantify syringic acid. Samples were typically approximately 100 pl volume. The analysis of rhizosphere and plant cells in micro scale (pico and nano-litre) using CE are described in detail by Tomos (2015).

2.13 Phenolase assay

2.13.1 Microtiter plate assay

The degradation of phenol in the soil was investigated by measuring phenolase activity using a microtiter plate reader (Biotek Power Wave XS) at a wavelength of 250 nm. A set of sample solution with concentrations ranging from 0.5 to 10 mM of phenol was used to generate a calibration curve from absorbance values. Soil solution (obtained as described in section 2.8.1) as source of enzyme was added to equal amount of phenol solution as substrate (see section 6. 3). Phenol concentration was determined at room temperature at various intervals.

2.13.2 Spectrophotometer assay

A spectrophotometer (Jenway 6405 UV / Vis) was used to determine phenol concentration using a glass cuvette instead of the plastic plate in the microtiter plate assay (to avoid any interaction of phenol with plastic). Phenol analysis was carried out by measuring the absorbance at 250 nm (Thomasa & Theraulazb, 2007). A standard calibration curve was previously constructed using phenol concentration ranging from 1 to 10 mM. This standard curve was used to determine the concentration of phenol.

2.14 Data analysis

Data analyses were performed through IBM SPSS Statistic version 22 for Windows. Results include the means and the standard error. A statistic analysis of data was carried out on the assumption of normal distribution and homogeneity of variance. Treatment effect were tested using analyses of Tukey multiple comparison. The significance level was set at $P = 0.05$. This analytical methods was used in all relevant experiments.

Chapter 3: Decomposition of phenolic compounds in soil in the presence and absence of plants.

3.1 Introduction

In bioremediation and phytoremediation, it has been found that plant roots and microbes play an important role in the removal of organic pollutants (De Araujo *et al*., 2002; Kang, 2014). This chapter was aimed at estimating the removal of different phenolic compounds from the soil in the absence of plants to assess the role of microbes. Then, in the presence of plants, to assess the root impact on the phenols removal using low resolution mapping. The behaviour of eight different phenolic compounds was determined after their addition to non-autoclaved and autoclaved (control) soil (section 3.2). A microcosm system was used to study the effect of two species, rye and wheat on the behaviour of phenolic compounds in the rhizosphere compared to their behaviour some distance away from root as a control (3.3). The concentration of phenolic compounds was measured using a capillary zone electrophoresis CZE technique (2.10) using a conventional (macro) approach to sample the soil (section 2.8.1).

Three quantitative parameters emerged as being important in the interpretation of the raw data. These are the dilution of the applied phenolics, an adsorption effect and the degradation processes. As expected, the initial applied concentration decreased immediately after addition to the soil at zero time. This was partially due to its dilution upon addition to moist soil. The magnitude of this can be estimated from the known moisture content of the soil (generally approximately (7%)) and the volume of phenol solution added. However, the observed decrease was generally considerably more than this. It was concluded that this additional effect was due to adsorption processes in the soil. An Adsorption Ratio was defined as the fraction of the expected diluted concentration that was actually measured:

$$\text{Adsorption Ratio} = \frac{\text{Measured Concentration}}{\frac{(\text{Initial Concentration} \times \text{Volume added})}{(\text{Volume added} + H_2O \text{ Volume in soil})}} \qquad \textit{Equation } (3.1)$$

The behaviour of the residual free phenolic was the subject of the bulk of this chapter and thesis.

3.2 The degradation of phenolic compounds from extractable moisture in non-autoclaved and autoclaved soil

The disappearance of phenolics after their addition into soil could mean degradation, adsorption or evaporation.

3.2.1 Dilution and adsorption

Each of the selected phenolics (10 ml) was added separately to 100 g of non-autoclaved and autoclaved soil (section 2.4). The soil had been autoclaved before the addition of phenol (2 .3). The soil, contained 7 ml of water (section 2.2). Therefore, when this 10 ml of 20 mM phenol was added to 100 g of soil of 7% moisture content, the concentration of phenol after mixing would be expected to be 11 mM. However, the most rapid measurement (taken after approximately 10 minutes), gave a concentration of 4.5 mM. This suggests that more than simple dilution was occurring at this point in the protocol.

To describe this phenomenon, an "adsorption ratio" was calculated as the ratio of the observed and expected concentrations (due to dilution). In the case of phenol in non-autoclaved soil this was 0.38 (Table 3.1).

	Non-Autoclaved soil			Autoclaved soil		
Compounds	Adsorption Ratio	$t_{½}$ (hours)		Adsorption Ratio	$t_{½}$ (hours)	
		Slow phase	Rapid phase		Slow phase	Rapid phase
Phenol	0.38	13.4	3	0.47	433	3
2,4DNP	0.16	-	-	0.16	-	-
3CP	0.20	577	3	0.13	693	-
2CP	0.20	15	3	NA	NA	NA
Vanillic acid	0.17	-	0.30	0.58	3	-
Syringic acid	0.35	-	3	0.74	24	3
Ethylvanillin	0.30	-	1.9	0.40	96	3
DMHAP	0.29	-	3	0.43	103	-

Table 3.1 The half-life of four artificial and four biological phenolic compounds measured in bulk autoclaved and non-autoclaved soil without plants. There is a range and sterilization increase $t_{½}$ in all cases. Results show that the adsorption ratios were higher for most chemicals tested in autoclaved soil.

3.2.2 Phenol

Phenol (20 mM) was mixed with non-autoclaved and autoclaved soil. The concentration of phenol in moisture of the soils was measured at various time up to 2 days. Phenol depletion occurred differently in non-autoclaved and autoclaved soil (Figure 3.1).

Several feature can be noted. At first glance the phenol seems to present an exponential decay (dotted line). The half time of this, 13.4 hours, was calculated from a linear regression (Table 3.1). Autoclaved soil (Figure 3.1B) presented an even more complex picture. Following, the "adsorption" effect, a short exponential decrease was noted (dotted line). This, however, did not decay to zero concentration and appears to be a different phenomenon to that observed in Figure 3.1 A. This was interpreted as indicating two decay phases; a rapid phase with a $t_{½}$ of approximately 3 hours and a much slower phase ($t_{½}$ = 433 hours) (Table 3.1). Closer inspection of Figure 3.1A (non-autoclaved) indicated that it also may represented the same two decay phases with a rapid phase ($t_{½}$ = 5 hours) superimposed on a longer one that decays to zero. In this case, the longer $t_{½}$ is properly close to 30 hours.

3.2.3 3-Chlorophenol

Similar observations were made for 3-Chlorophenol (3CP) after the addition of 20 mM soil (section 2.4). Here, however, the slow degradation phase was very much longer with a $t_{½}$ > 500 hours in both autoclaved and non-autoclaved soil (Figure 3.2, Table 3.1). The dilution and adsorption phenomena are the same as for phenol. However, the rapid phase of $t_{½}$ (3 hours) was only seen in the non-autoclaved soil (Figure 3.2 A, Table 3.1). The adsorption ratio was 0.2.

3.2.4 2-Chlorophenol

Only data for non-autoclaved soil is (Figure 3.3) available for 2-Chlorophenol (2CP) (using 20 mM). Its behaviour resembles that of phenol with a $t_{½}$ of 15 hours rather than 3CP ($t_{½}$ = 577 hours) (Table 3.1). While the concentration of 2CP at zero time was < 2 mM. Although, like phenol, it is possible that a rapid phase with a $t_{½}$ of approximately 2 hours can also be seen (Figure 3.3). The adsorption ratio was 0.2 similar to 3CP.

3.2.5 2,4-Dinitrophenol

No equivalent time course was measured for 2,4DNP (2 mM) Figure 3.4. However, if it assumed that it has a similar adsorption ratio to all of the previous compounds (Table 3.1), it would appear to have t½ of 10 minutes needed to take the first sample for both autoclaved and non-autoclaved soil. The argument for this is the same as for vanillic acid in non-autoclaved soil below. The implication of this is that breakdown of 2,4DNP is non-microbial, or that it's degrading microbes can withstand autoclaving. Any additional microbial breakdown cannot be observed as it would be masked by this apparent rapid non-microbial activity.

3.2.6 Ethylvanillin

Similar observations were made for ethylvanillin (where 10 mM was used) (Figure 3.5 and Table 3.1). The behaviour was qualitatively similar to phenol. The rapid phase, however, was even faster with a t½ of approximately 3 hours. The slow phase observed under autoclaved conditions had a t½ of 96 hours. Since the rapid phase in non-autoclaved soil totally depleted the ethylvanillin, no slow phase could have been detected. The adsorption ratio was similar to that of phenol (Table 3.1).

3.2.7 3, 5-Dimethoxy-4-hydroxyacetophenone

Similar observation were made for DMHAP (again using 10 mM) (Figure 3.6 and Table 3.1). In this case, however, no obvious slow phase was seen for the non-autoclaved soil (Figure 3.6 A). The t½ was approximately 3 hours. In autoclaved soil, no obvious rapid phase was seen with the slow phase having a t½ of approximately 103 hours (Table 3.1). The adsorption ratio was 0.29 and 0.43 in autoclaved and non autoclaved soil respectively.

3.2.8 Vanillic acid

The results for vanillic acid (used at 2 mM) are different (Figure 3.7 and Table 3.1). Although very few data were collected for non-autoclaved soil, it is evident that all traces had disappeared by 3 hours after addition. This indicates a very rapid degradation with the adsorption ratio and t½ being impossible to measure. In autoclaved soil, the degradation is again slower (as seen for all solutes) but only to a rate equivalent to a t½ of approximately 3 hours. Here an adsorption ratio of 0.58 can be estimated. This is higher than for previous solutes.

3.2.9 Syringic acid

Syringic acid (used at 20 mM) resembles the behaviour of DMAP (Figure 3.8 and Table 3.1). In non- autoclaved soil was again observed a pattern that suggests a t½ of 3 hours. A relatively

rapid degradation was seen in autoclaved soil ($t_{½}$ = 3 hours) together a slow phase with a $t_{½}$ of approximately 24 hours. The adsorption ratio was 0.74. This is the highest value of all solutes.

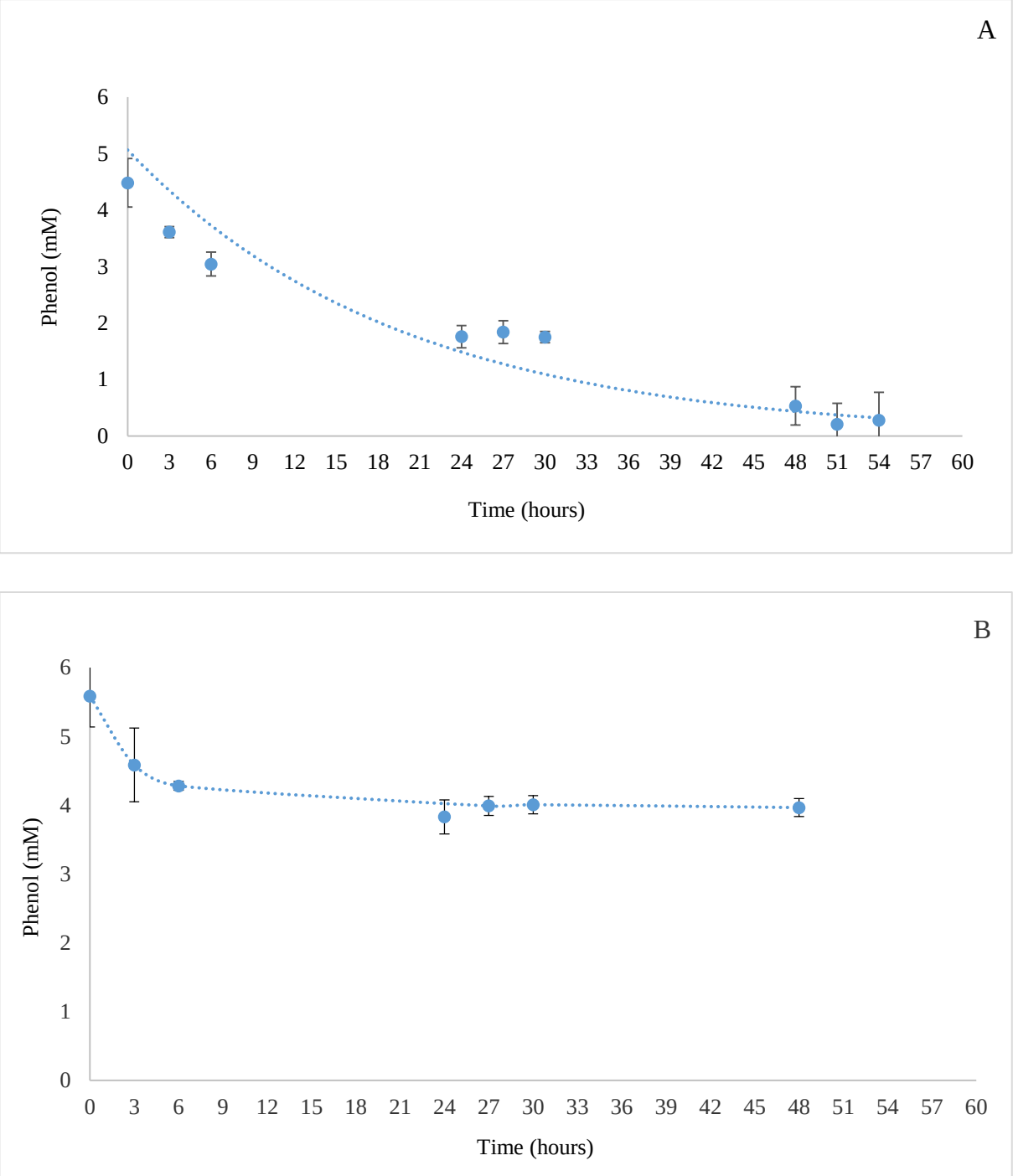


Figure 3.1 Phenol degradation over time in extractable soil moisture. Phenol (10 ml of 20 mM) of was added to 100 g normal (untreated) soil (A) and similar quantity of autoclaved soil (B). Phenol degradation over time is shown to differ depending on the soil used. There was a dramatic reduction of phenol concentration in non-autoclaved soil over 54 hours time (A). The concentration in the autoclaved soil had very minimal reduction (B). Autoclaving has significant effect on reduction of phenol concentration at 24, 27, 30 and 48 hours compared to non-autoclaved soil at the same duration of time ($P< 0.05$). Between 30 to 48 hours duration phenol concentration is seen to reduce very rapidly in non-autoclaved soil $P< 0.05$ (rapid phase). Meanwhile the duration of time has no effect on the phenol content of autoclaved soil $P> 0.05$(slow phase).This suggests some biological factor in the

soil was contributing to the rapid degradation of phenol. Three other phenomena were also noted. The dilution of the added phenol by soil and an instantaneous "absorbance" of phenol by a solid phase and two exponential decay processes (Table 3.1) (see text for details). Error bars show ± standard error (SE) (n = 3).

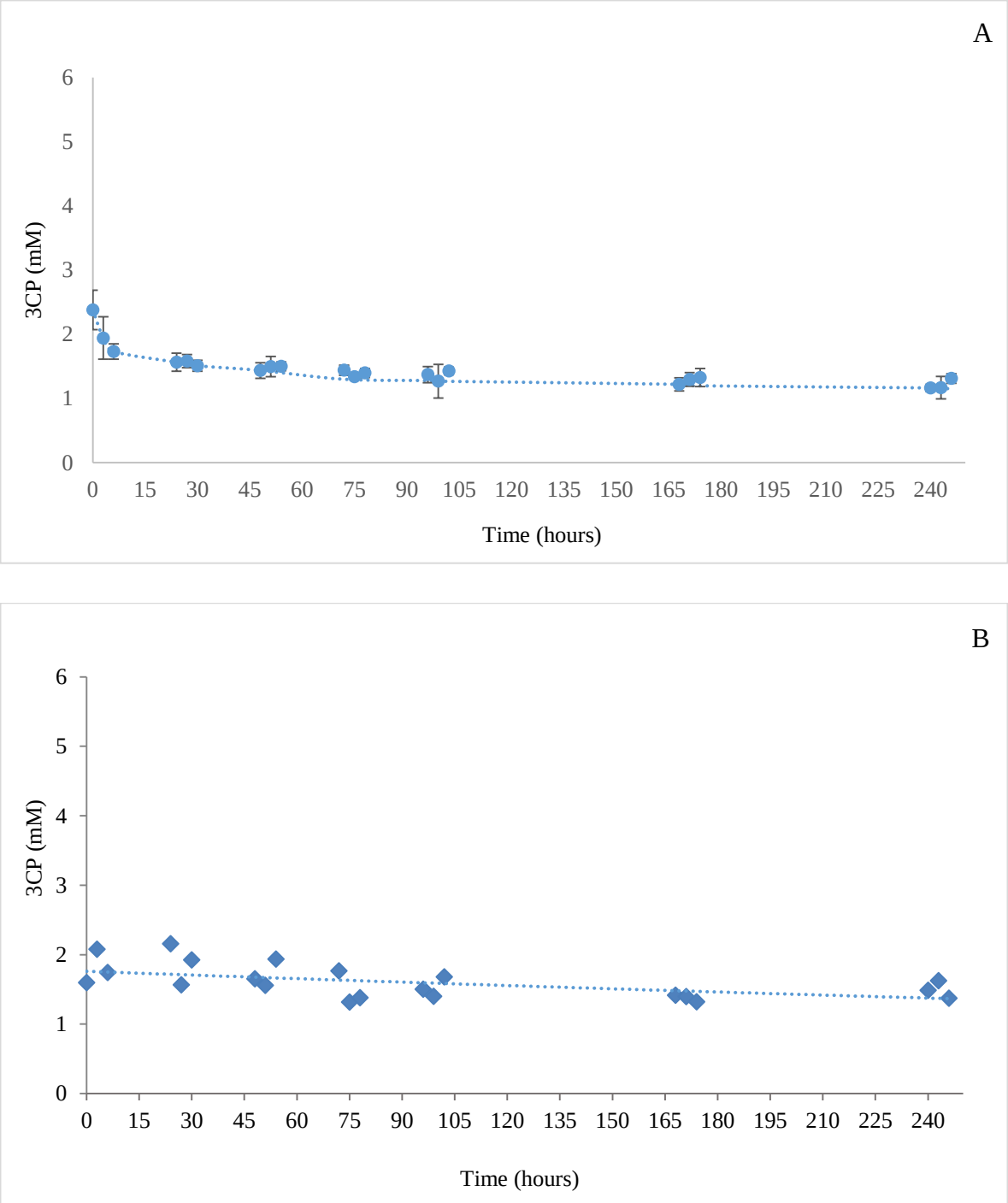


Figure 3.2 3-Chlorophenol degradation over time in extractable soil moisture. Phenol (10 ml of 20 mM) of was added to 100 g normal (untreated) soil (A) and similar quantity of autoclaved soil (B).The concentrations and conditions were similar to those in Figure 3.1, except the experiment was run for 250 hours. The dilution, adsorption and two decay processes were again found; although they differ in detail from those of phenol (Table 3.1). (See text). Error bars show ± (SE) (n = 3).

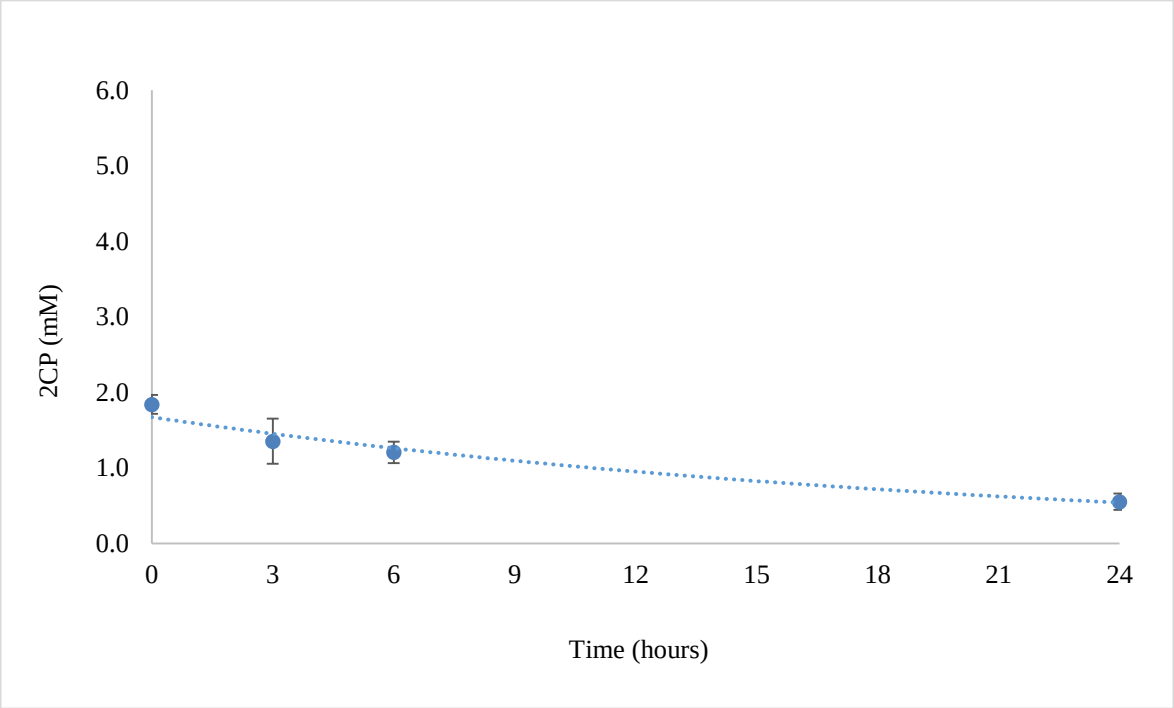


Figure 3.3 2-Chlorophenol degradation over time in extractable soil moisture. 2CP (20 mM) was added to non-autoclaved soil. The concentration was analysed at regular intervals from 0 to 24 hours. The 2CP was detected even at 24 hours but significantly reduced in concentration. Error bars show ± (SE) (n = 3).

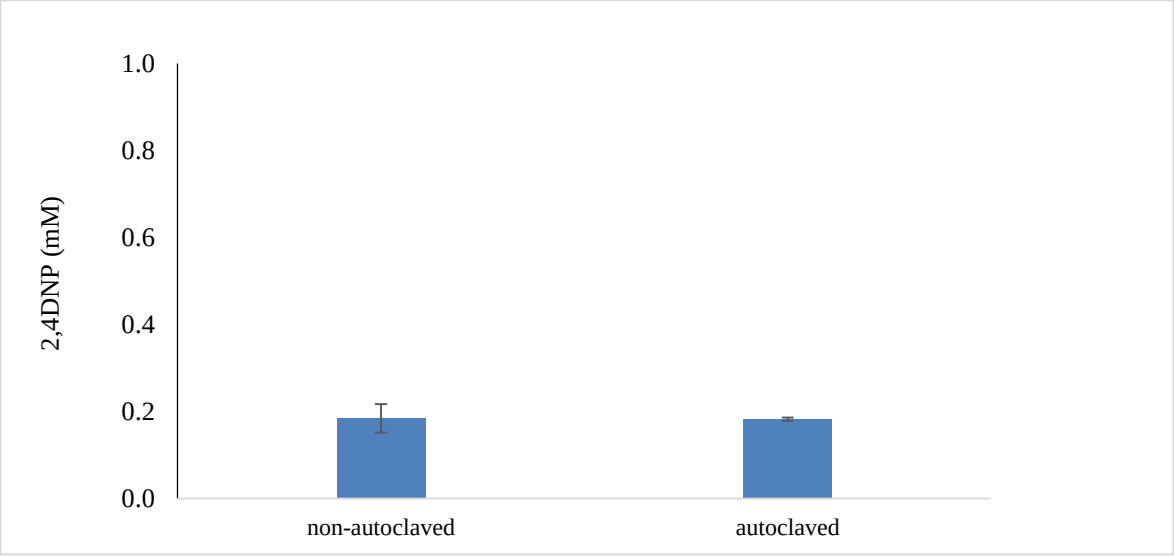


Figure 3.4 2,4DNP disappearance in extractable soil moisture. 2,4DNP 10 ml of (2 mM) of the chemical was added in non-autoclaved and autoclaved soil. The samples was taken from each of at 0 time (10 minutes of the addition) and analysed for 2,4DNP concentration. The results show there is no significant difference between the two. Error bars show ± (SE) (n = 3).

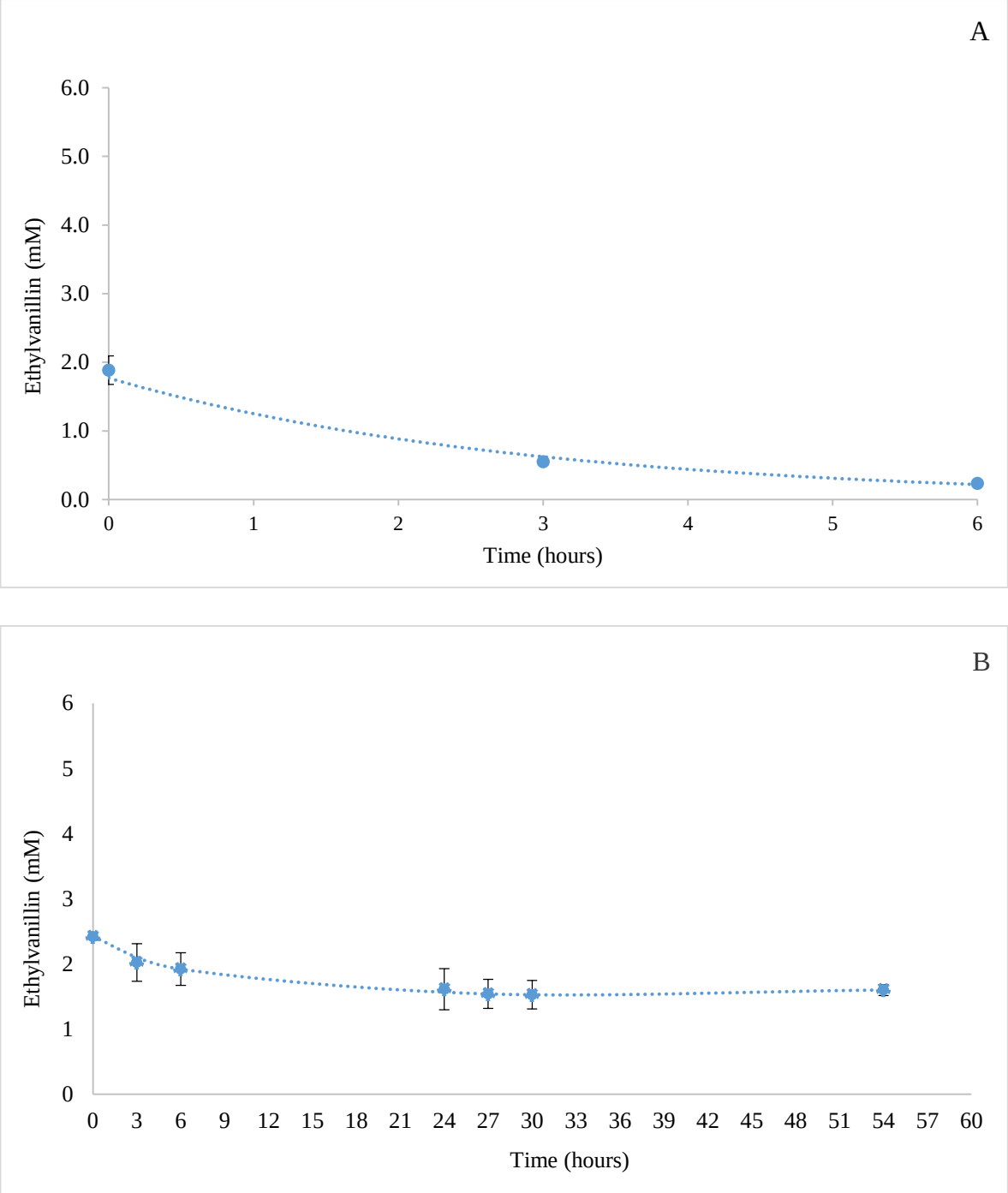


Figure 3.5 Ethylvanillin degradation over time in extractable soil moisture. Ethylvanillin (10 ml of 10 mM) was added into 100 g of normal (untreated) soil (A) and similar quantity of autoclaved soil (B). Similar results to those for phenol were observed for all phenomena (Table 3.1) except that no slow degradation phase was seen in non-autoclaved soil (Figure 3.5 A). Error bars show ± (SE) (n = 3).

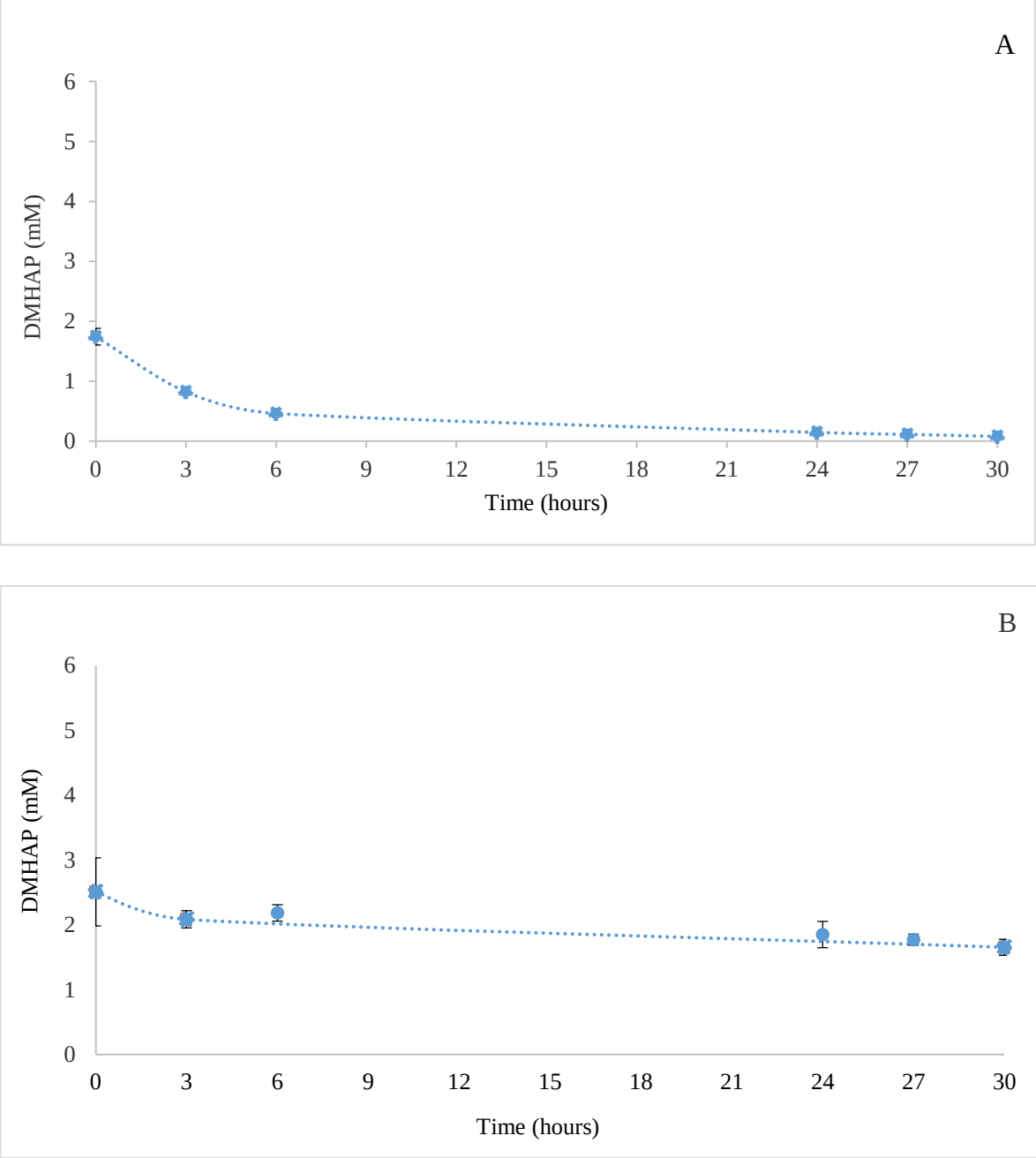


Figure 3.6 DMHAP degradation over time in extractable soil moisture. DMAP 10 ml of (10 mM) was added in 100 g non-autoclaved soil and 100 g soil that was autoclaved prior to inoculation of DMAP. Soil samples were analysed at regular intervals to determine the concentration for a period of 30 hours. DMAP concentration decreases gradually until it was almost undetectable at 30 hours (A). In the autoclaved soil, there seemed to be no significant depletion of the chemical (B) $P > 0.05$. Error bars show ± (SE) (n = 3).

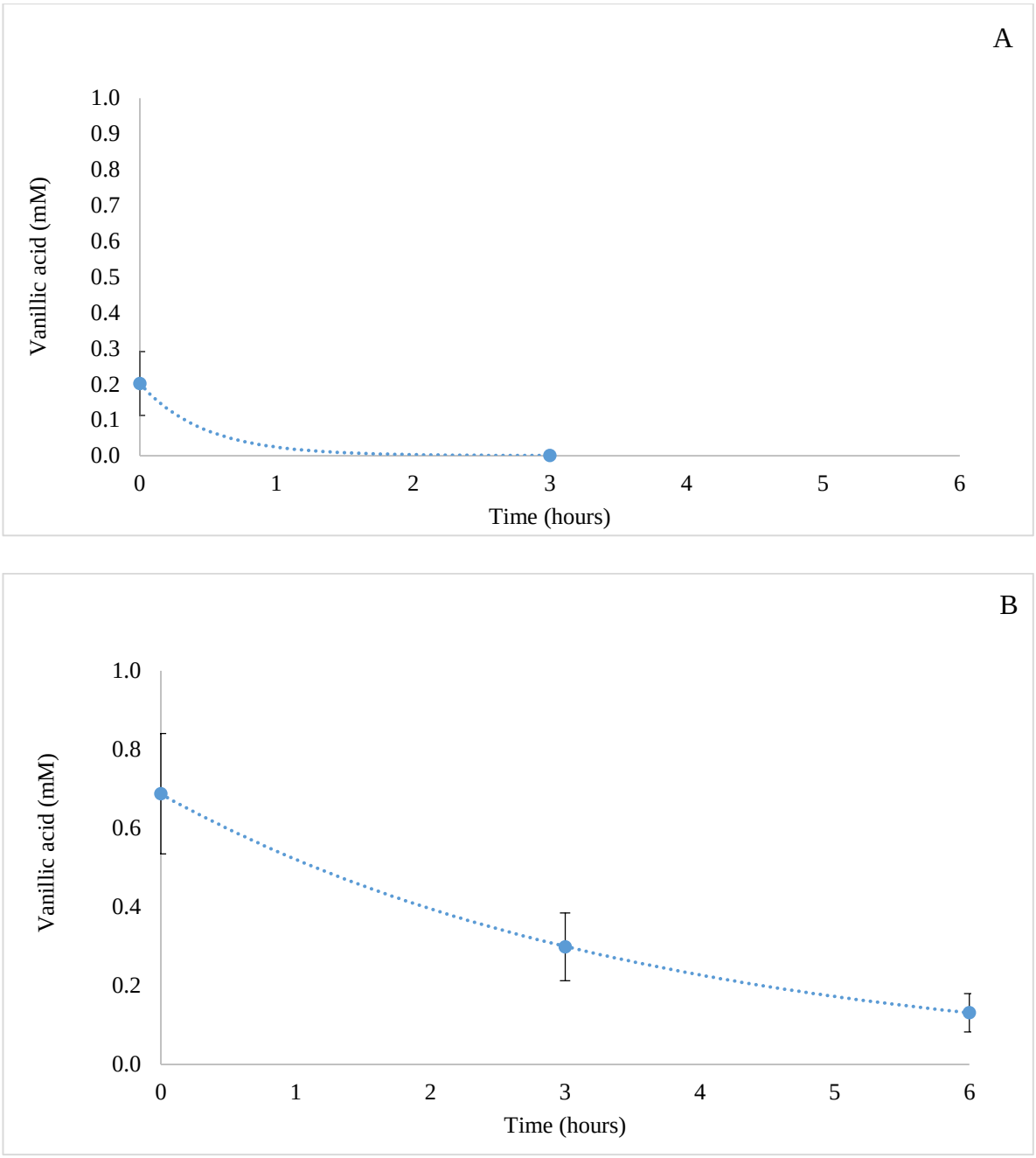


Figure 3.7 Vanillic acid degradation over time in extractable soil moisture. Vanillic acid 10 ml of (2 mM) was added in 100 g non-autoclaved soil and 100 g soil that was autoclaved prior to inoculation with vanillic acid. Soil samples were analysed at regular intervals to determine the concentration for a period of 6 hours. Vanillic acid concentration dramatically decreased to undetectable level just after1 hour in non-autoclaved soil (A). In the autoclaved soil, the concentration of vanillic acid decreases gradually and was still at detectable level (approximately 0.2 mM) at 6 hours (B). Error bars show ± (SE) (n = 3).

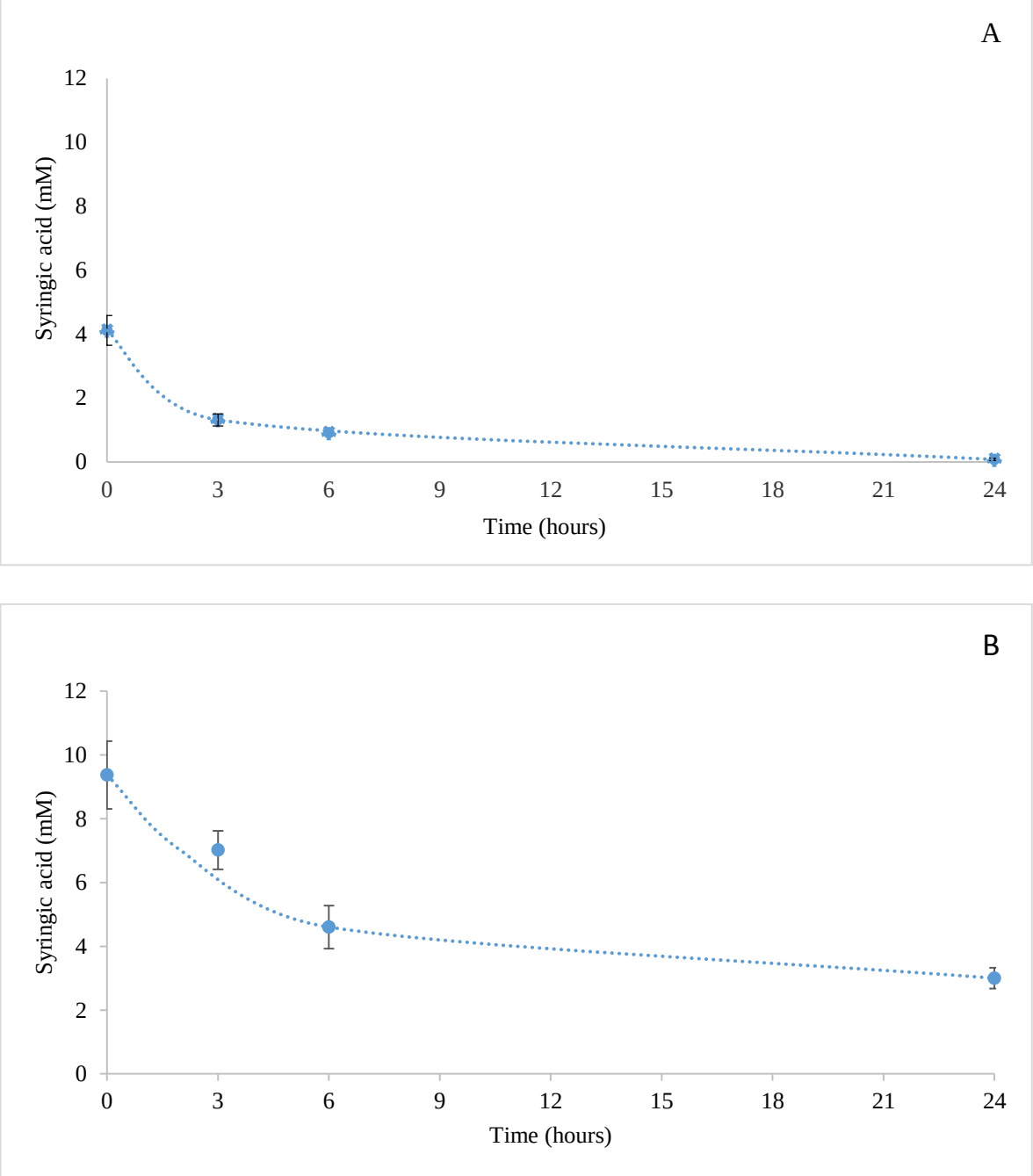


Figure 3.8 Syringic acid degradation over time in extractable soil moisture. Syringic acid 10 ml of (20 mM) was added in 100 g non-autoclaved soil and 100 g soil that was autoclaved prior to inoculation with syringic acid. Soil samples were analysed at regular intervals to determine the concentration over a period of 24 hours. Syringic acid concentration dramatically decreased. After 24 hours it became undetectable (A). In the autoclaved soil, the concentration of syringic acid decreases gradually and was still at detectable level (approximately 3 mM) at 24 hours (B). Error bars show ± (SE) (n = 3).

3.2.10 Comparison of adsorption ratio of different phenolic compounds

The adsorption ratio was compared between non-autoclaved and autoclaved conditions in order to distinguish between the biotic and abiotic effect on degradation of phenolic compounds in the soil (Figure 3.9). Generally, two features were observed. Firstly, the autoclaved soil had a significantly higher adsorption ratio ($P< 0.05$) than the non-autoclaved one for most solutions tested with the exception of 2,4DNP (3CP had a significantly lower ratio). There was difference in the adsorption ratio between phenolic, syringic and vanillic acids. That may be due to varying specific adsorption site for each of them in the soil. In autoclaved condition, elimination of living microbes may release more surface area.

Secondly, it is noted that increase in the adsorption ratio is proportionate to the increase in the concentration of the phenolic compounds; with exception of 3CP and DNP. In general, most of the adsorption ratio was in the range between 0.3 to 0.4 in non autoclaved soil and between 0.1 to 0.7 in autoclaved soil.

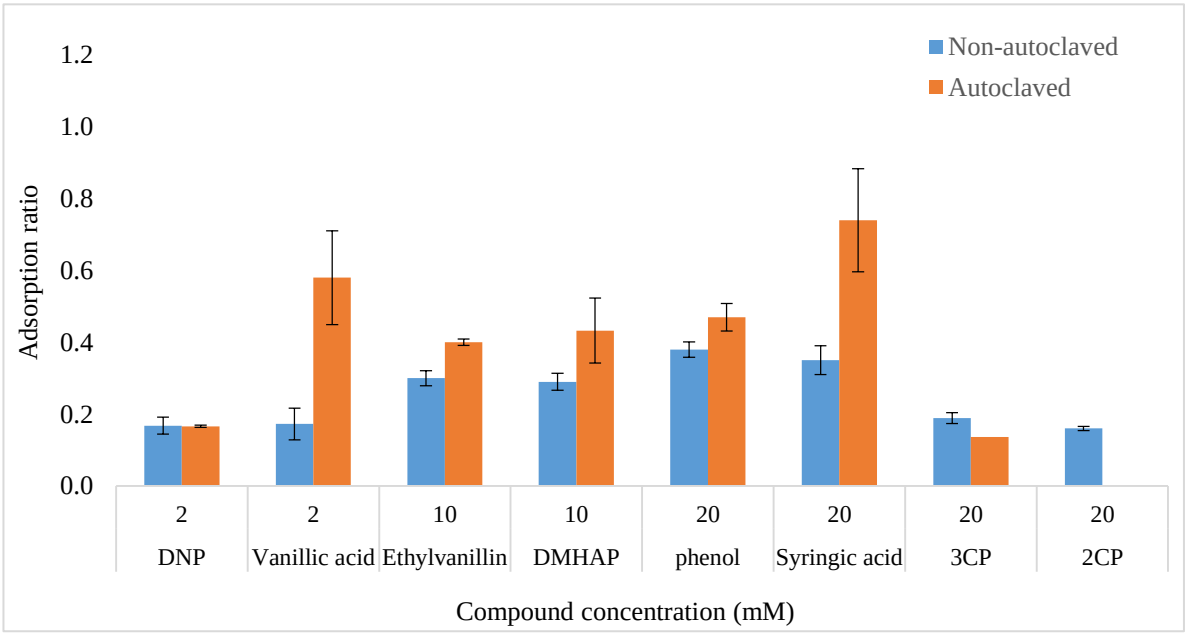


Figure 3.9 Comparison of adsorption ratio of different phenolic compounds in autoclaved and non-autoclaved soil. This is the data for Table 3.1 set out in the order of concentration of solutes applied. The adsorption ratio was higher in autoclaved samples for all compounds than in non-autoclaved samples with the exception of 2,4DNPand 3CP.

3.3 The disappearance of phenolic compounds from soil in the presence of plants (rye and wheat)

Non-autoclaved soil was shown to promote degradation of phenolic compound in our previous experiments. We wanted to examine if plants could also contribute to the degradation process in soil. Rye and wheat plants were used in the experiment. In contrast to the previous section in which 100 g sample of soil were mixed with the tested phenolics, rye or wheat plants were grown in microcosms (section 2.6.1) and a 5 ml sample of 20 mM concentration of a range of phenolic compounds was added by spray (section 2.7.1) into each microcosm. A sample (approximately 0.5 g) of soil close to the root (≤ 1mm) and at the bottom of the microcosm (> 2 cm away from the visible root) was collected (section 2.8.1; macro approach) at 0, 3, 6 and 24 hours to determine the concentration of the phenolic compound over time. Graphs were plotted and the $t_{½}$ was determined.

3.3.1. Phenol

The behaviour of phenol in the soil away (>2 cm) from root surfaces / outer layer of the root was similar to that described in the previous section (Figure 3.1 A; non-autoclaved in the absence of plants) (Figure 3. 10B for rye and Figure 3.11B for wheat). (Although different quantities of soil and phenol solution were use, the proportions were identical.) The dilution and adsorption phenomena were again observed (Figure 3.10B and 3.11B) with the adsorption ratio being similar (Table 3.2 – corresponding to Table 3.1; Figure 3.12-corresponding to Figure 3. 9).The data in these figures also suggest that both fast and slow degradation phases were again seen. The estimated $t_{½}$ values are shown in Table 3. 2.

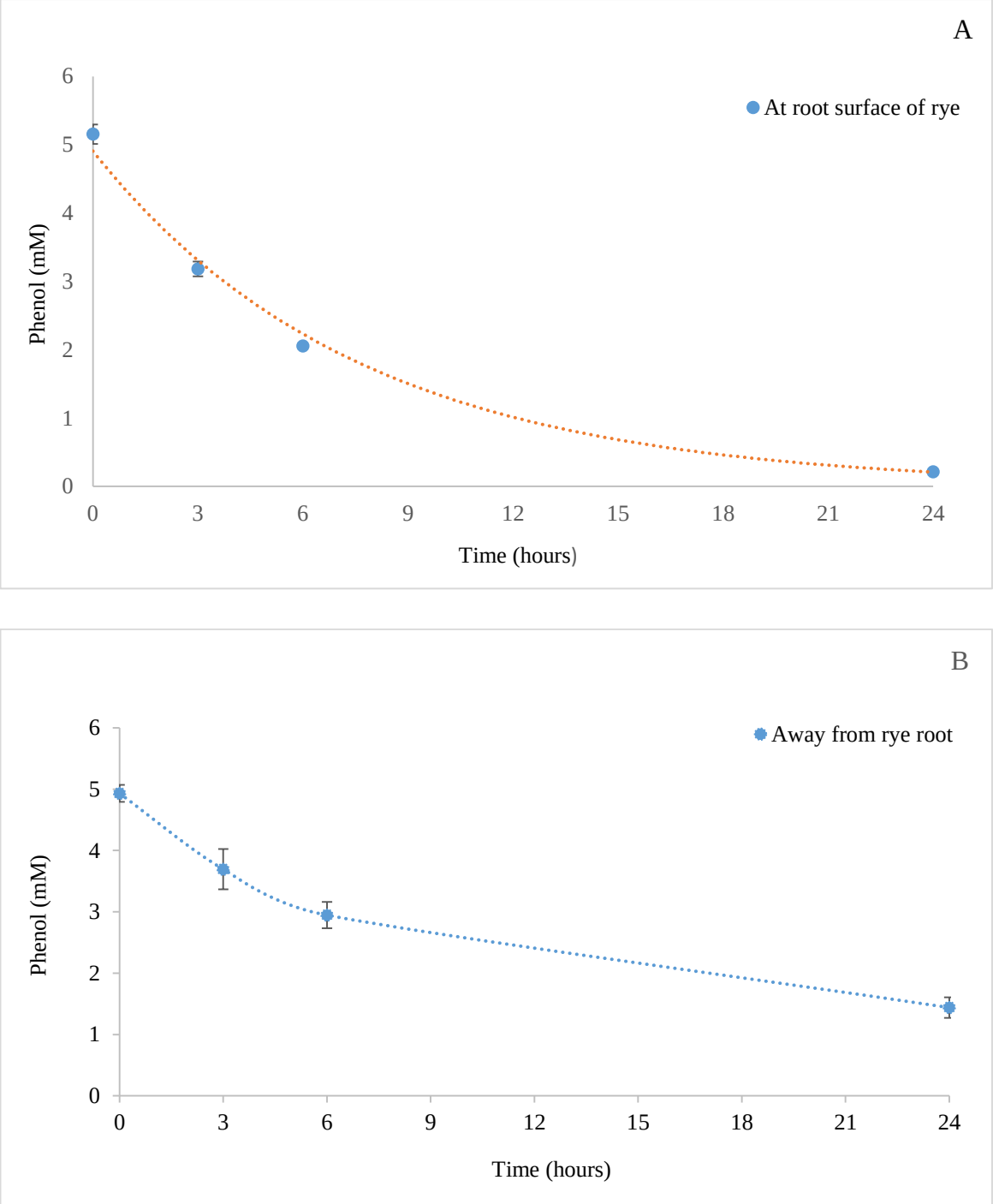


Figure 3.10 Concentration of phenol in soil extractable moisture was measured over time in presence of rye plants. Soil samples were collected from around (≤ 1mm) the root (A) and away (>2cm) from the root (B) in the microcosm. Concentration of phenol from soil collected from around the root decreased dramatically to undetectable level in 24 hours (A). Phenol concentration in soil samples collected away from the plant was shown to deplete at much slower rate with the phenol still present after 24 hours (B). Error bars show ± (SE) (n = 9).

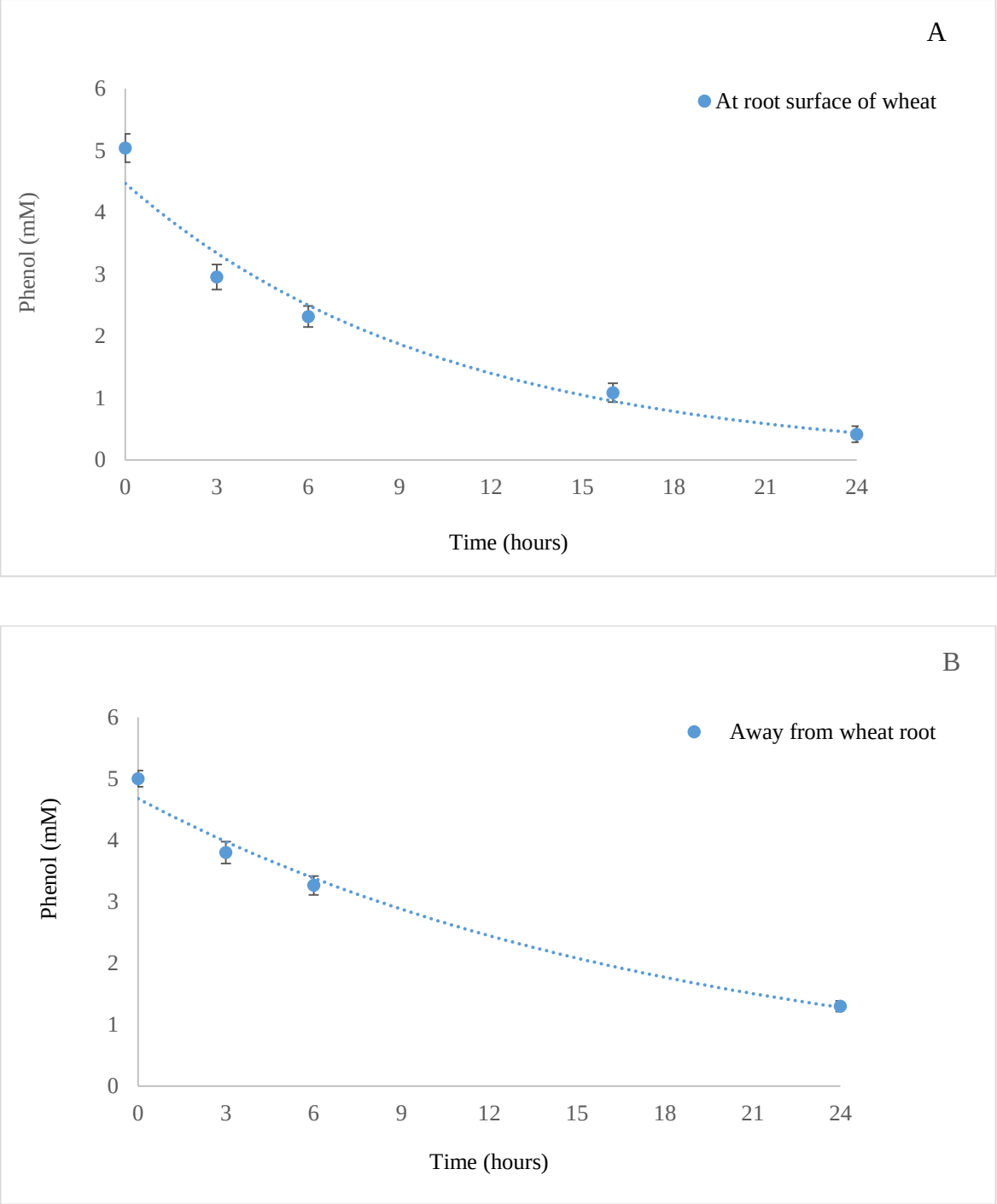


Figure 3.11 Similar experiment to Figure 3.10 (but for wheat). Concentration of phenol in soil extractable moisture was measured over time in presence of wheat plants. Soil samples were collected from around (≤ 1mm) the root (A) and away (>2cm) from the root (B) in the microcosm. The concentration of phenol in the presence of wheat appears to be rapidly reduced in 24 hours (A) compared to soil sample collected away from the plants roots, which contains significantly high concentration phenol even after 24 hours (B). Error bars show ± (SE) (n = 9).

A (Rye)	Away from root			At root surface		
	Adsorption Ratio	Slow Phase $t_{½}$ (hours)	Rapid Phase $t_{½}$ (hours)	Adsorption Ratio	Slow Phase $t_{½}$ (hours)	Rapid Phase $t_{½}$ (hours)
Phenol	0.5	17	2	0.5	6	2
3CP	0.2	130	2	0.2	10	3
2CP	0.2	17	2	0.2	9	3

B (Wheat)	Away from root			At root surface		
	Adsorption Ratio	Slow Phase $t_{½}$ (hours)	Rapid Phase $t_{½}$ (hours)	Adsorption Ratio	Slow Phase $t_{½}$ (hours)	Rapid Phase $t_{½}$ (hours)
Phenol	0.5	13	1	0.5	8	2
3CP	0.2	150	2	0.2	13	2
2CP	0.2	15	2	0.2	12	2

Table 3.2 Adsorption ratio of phenolic compounds in soil microcosm in the presence of rye (A) and wheat (B). The $t_{½}$ of slow phase and rapid phase for each compounds.

The slow phase $t_{½}$ values (17 and 13 hours for rye and wheat) are very similar to that (13.4 hours) in Table 3.1. The fast phase values are somewhat shorter (2 and 1 hours) than in Table 3.1 (2.5 hours). However, it must be admitted that the extrapolations are based on very limited data and it is unlikely that these differences are significant.

The behaviour of phenol in the soil close ($\leq$1mm) to root surfaces is illustrated in Figure 3.10A and 3.11A. Again a dilution/adsorption effect was observed. There was no significant difference in the adsorption ratio of phenol in soil at the root surface and away from the root (Figure 3.12). It is also similar to that in non-autoclaved soil (Figure 3.9). Again there is evidence for both a fast and a slow phase of phenol disappearance. Unfortunately, the nature of the data makes it impossible to determine statistically whether this was different for the soils away from and close to root surfaces (Table 3.2). The value of the slow phase, however, is arguably reduced in the vicinity of the root. A statistical analysis is impossible, but the values of phenol concentration in the soil moisture at 24 hours are statistically different between the two locations in both rye and wheat (Table 3.3). Moreover, rye was faster to degrade the phenol with only $t_{½}$ of 6 hours compared to wheat with $t_{½}$ of 8 hours. It appears that both rye and wheat

plant show very similar rate of degradation of phenol in soil although there was a different between them.

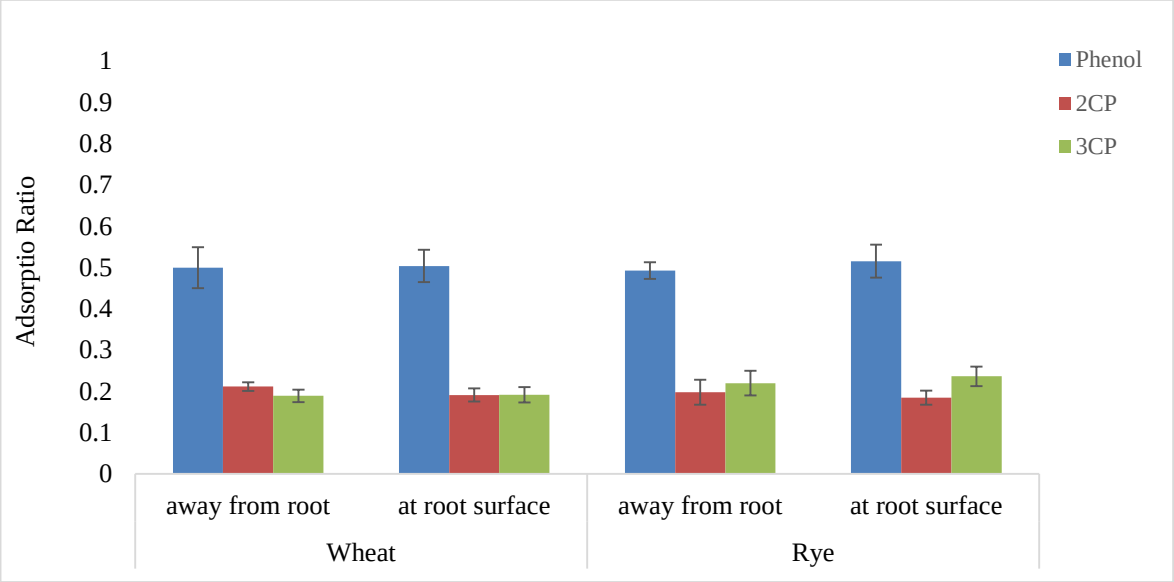


Figure 3. 12 Adsorption ratio of 20 mM phenolic compounds added to soil microcosm in the presence of rye and wheat in two different location, at root surface and away from root. There was not any significant difference in the adsorption ratio between the samples away from the root and the sample at root surface ($P > 0.05$) for all these compounds.

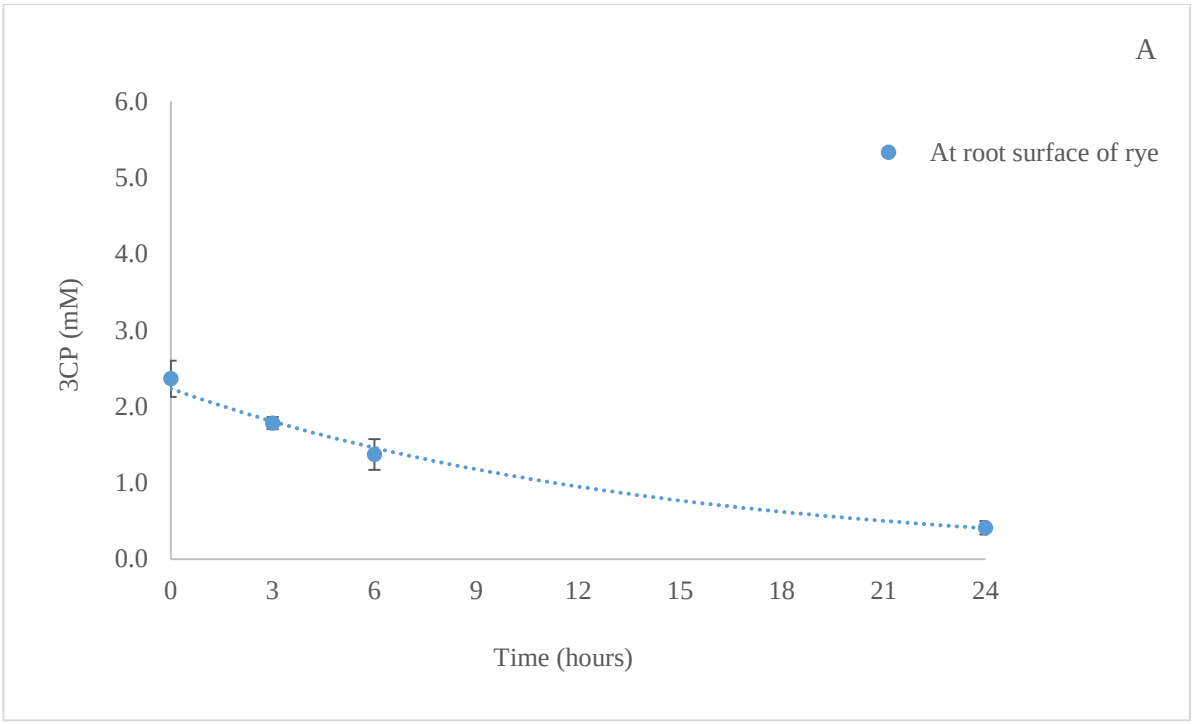
	Wheat				Rye			
Phenolic Compounds	away from root		at root surface		away from root		at root surface	
	Mean (mM)	SE	Mean (mM)	SE	Mean (mM)	SE	Mean (mM)	SE
Phenol	1.31*	0.06	0.42*	0.02	1.44*	0.05	0.07*	0.02
3CP	1.04*	0.03	0.53*	0.01	1.31*	0.04	0.41*	0.07
2CP	0.64*	0.02	0.46*	0.01	0.67*	0.03	0.28*	0.02

Table 3.3 The remaining concentration of 20 mM phenolic compounds added to soil microcosm with rye and wheat in two different location (away from root and at root surface) at 24 hours. Each data point represents the average of nine replicate and SE. The remaining concentration of phenol, 2CP and 3CP in soil samples at the root surface compared with remaining concentration of each compounds away from the root for both plants was significantly different* ($P < 0.05$).

3.3.2. 3-Chlorophenol

A similar experiment using rye and wheat was set. This time 3CP was tested in the microcosm. There was no different between 3CP in the soil away from the root in the soil microcosms from that of the previous experiment (Figure 3.2A; non-autoclaved in the absence of plants) for both rye and wheat (Figure 3.13B and Figure 3.14B). In addition, the effect of a dilution and adsorption phenomena was considered and it was similar in two different location in microcosm for rye and wheat (Table 3. 2). The $t_{½}$ of the fast phases was 2 hours and 1.7 hours respectively. Meanwhile, the $t_{½}$ of the slow phases was similar to that in non-autoclaved soil without a plant.

Results show that 3CP compound degraded faster close to the root of rye and wheat, effectively with half-life of approximately 10 and 13 hours in slow phase for rye and wheat respectively (Figure 3.13A and 3.14A; Table 3.2). The fast phases were 2.5 hours and 2 hours respectively (Table 3.2). The data in Figure 3.12 shows that the adsorption ratio for rye and wheat was similar in soil at the root surface and away from the root. This finding is similar to data for 3CP in non-autoclaved soil (Table 3.1). However, there was a significant different between 3CP residual concentration at 24 hours for rye and wheat $P < 0.05$ (Table 3.3).



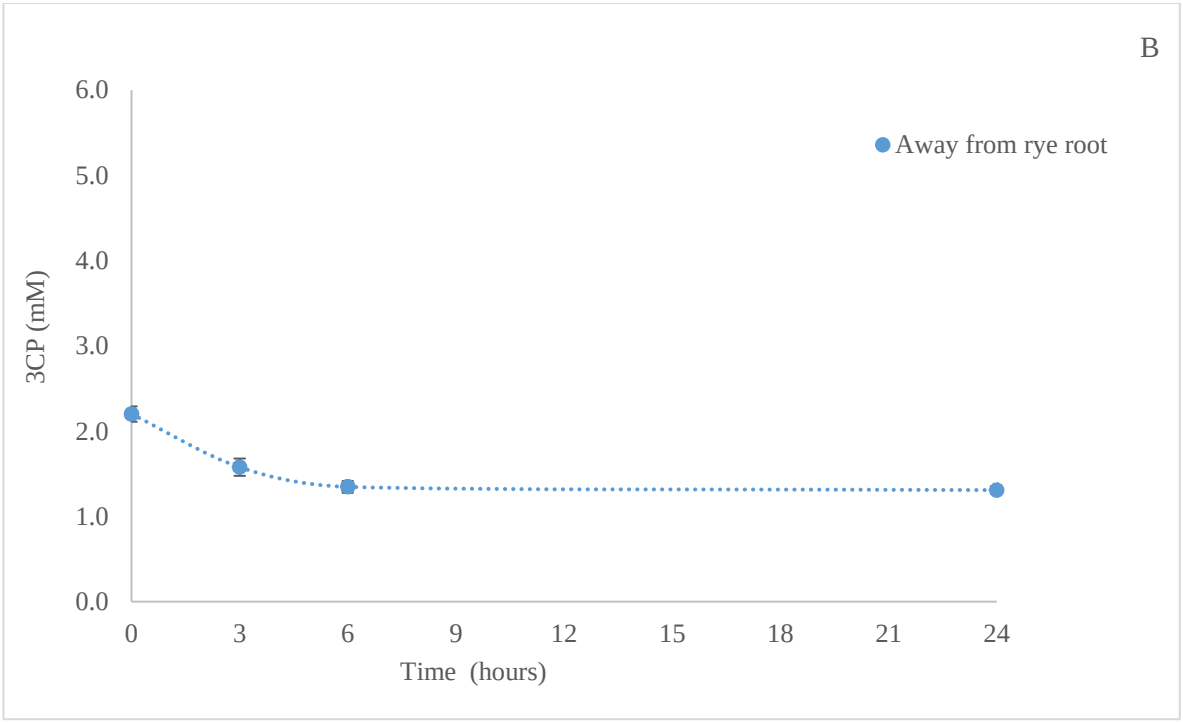


Figure 3. 13 Concentration of 3CP in soil extractable moisture was measured over time in the presence of rye plants. Soil samples were collected from around (≤ 1mm) the root (A) and away (>2cm) from the root (B) in the microcosm. The concentration of 3CP at root surface of rye appears to be rapidly reduced in 24 hours (A) compared to soil sample collected away from roots. This seems to reduce in concentration for first 6 hours then maintain significantly higher final concentration of 3CP even after 24 hours (B).(Mean ± SE, n = 9).

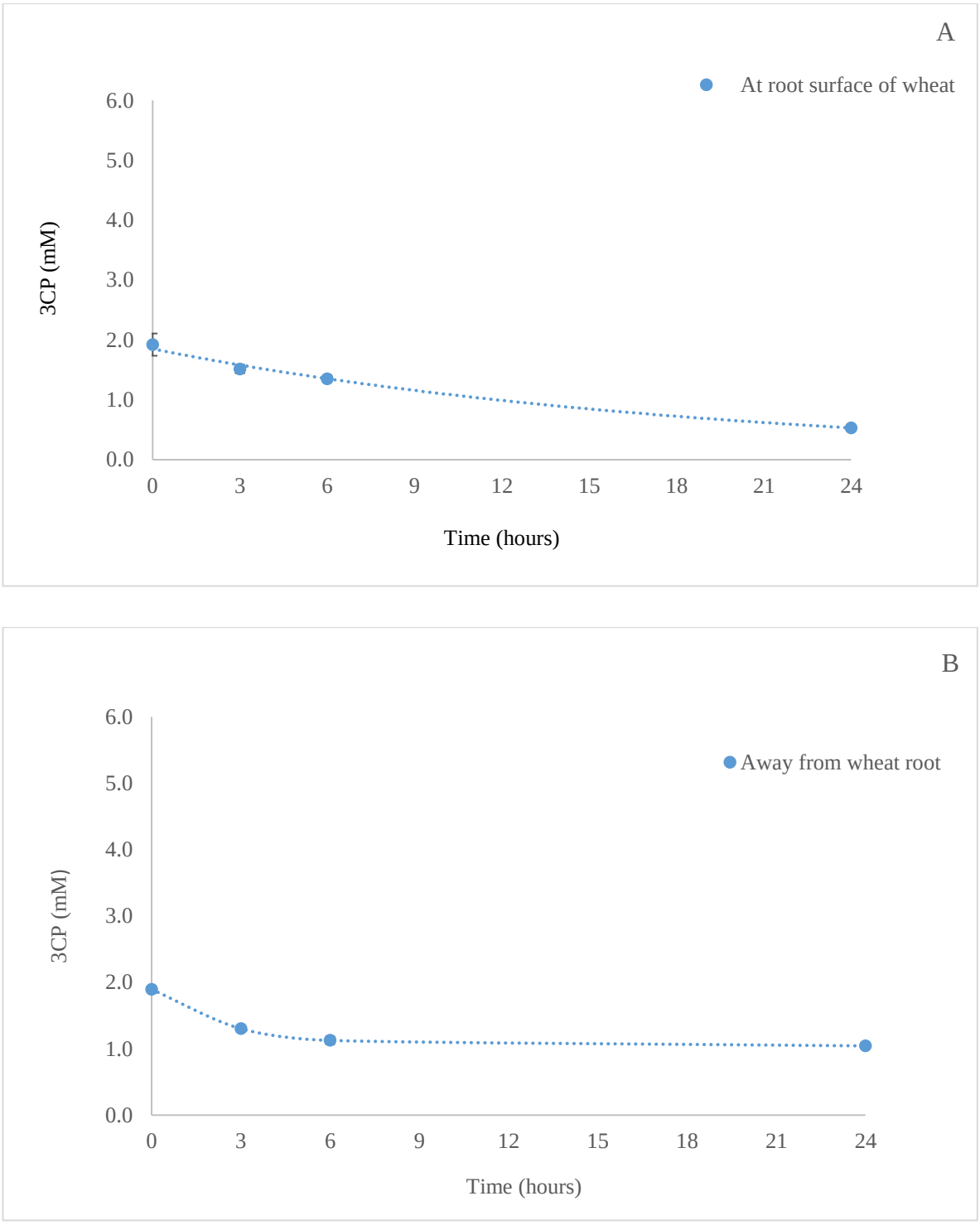


Figure 3.14 Concentration of 3CP in soil extractable moisture was measured over time in presence of wheat plants. Soil samples were collected from around (≤ 1mm) the root (A) and away (>2cm) from the root (B) in the microcosm. The concentration of 3CP in the soil microcosm near the root of wheat appears to be rapidly reduced in 24 hours (A) compared to soil sample collected away from roots, which initially reduced but still contains significantly higher in concentration of 3CP after 24 hours (B). (Mean ± SE, n = 9).

3.3.3 2-Chlorophenol

In experiments using 2CP, samples taken at the root surface of rye plants show a more rapid reduction in concentration compared to samples taken away from the root (Figure 3.15 and 3.16). In contrast, a similar ($t_{½}$) result was found between the samples taken away from root with non-autoclaved samples and with the absence of plant in the previous experiment (Figure 3.15 B and 3.16 B; Figure 3.3). The phenomena of dilution and adsorption were again observed (Table 3.2) and the results were similar for samples taken away from the root in the presence of both rye and wheat (Figure 3.12) and in the absence of plant as in previous experiment (Figure 3.9). Both rye and wheat result in a slow phase degradation of 2CP away from root. This shows no significant difference to the slow phase in soil without a plant (Table 3.2). Moreover, the fast phase ($t_{½}$) of 2CP in soil without a plant was about 1.8 hours; which was similar in both wheat and rye (Table 3.2). It was approximately 2.1 hours in rye and approximately 2 hours in wheat for the samples that were away from the root (Table 3.2).

The behaviour of 2CP in soil at the root surface in (Figure 3.15 A and 3.16 A) shows the same phenomena of dilution and adsorption ration (Table 3. 2), and seem to be similar for rye and wheat. The data in Figure 3.12 show adsorption ratios for both plants at the root surface. The fast phase was determined in rye and wheat at root surface. It was approximately 2.8 hours in rye and 1.9 hours in wheat.

The remaining concentration of 2CP in soil taken at the root of rye after 24 hours was approximately 0.3 mM. This was significantly different to the soil taken away from the rye root, which was approximately 0.7 mM (Table 3.3). The wheat plant shows shorter half-life of 2CP in soil samples collected at its root compared to the soil taken from away from the root. It appears that rye is better at degradation of 2CP compared to wheat.

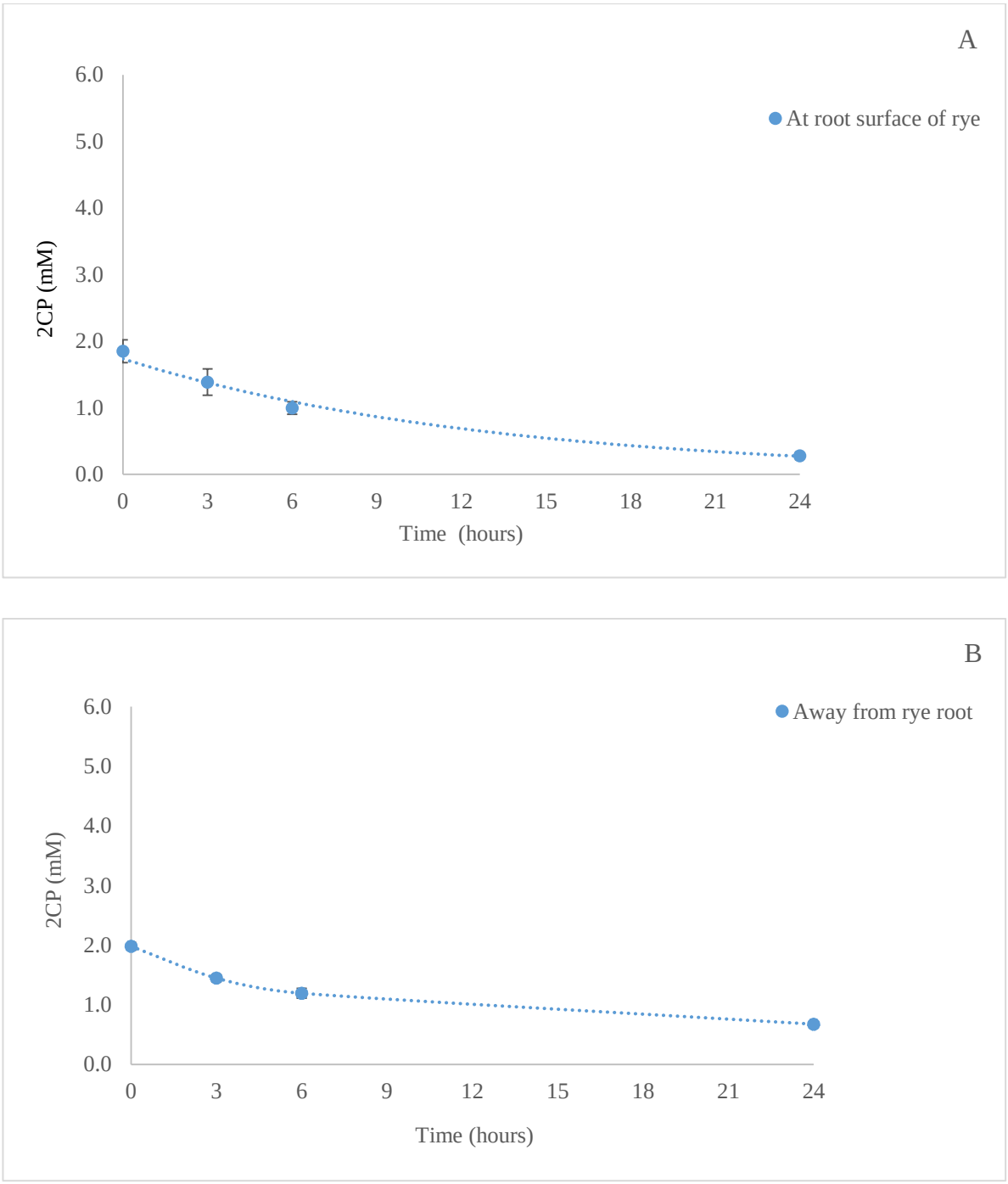


Figure 3.15 Concentration of 2CP in soil extractable moisture was measured over time in presence of rye plants. Soil samples were collected from around (≤ 1mm) the root (A) and away (>2cm) from the root (B) in the microcosm. The concentration of 2CP in the soil microcosm near the rye root is rapidly reduced in 24 hours (A) compared to soil sample collected away from the plants roots, which retains significantly higher concentration of 2CP at 24 hours (B). (Mean ± SE, n = 9).

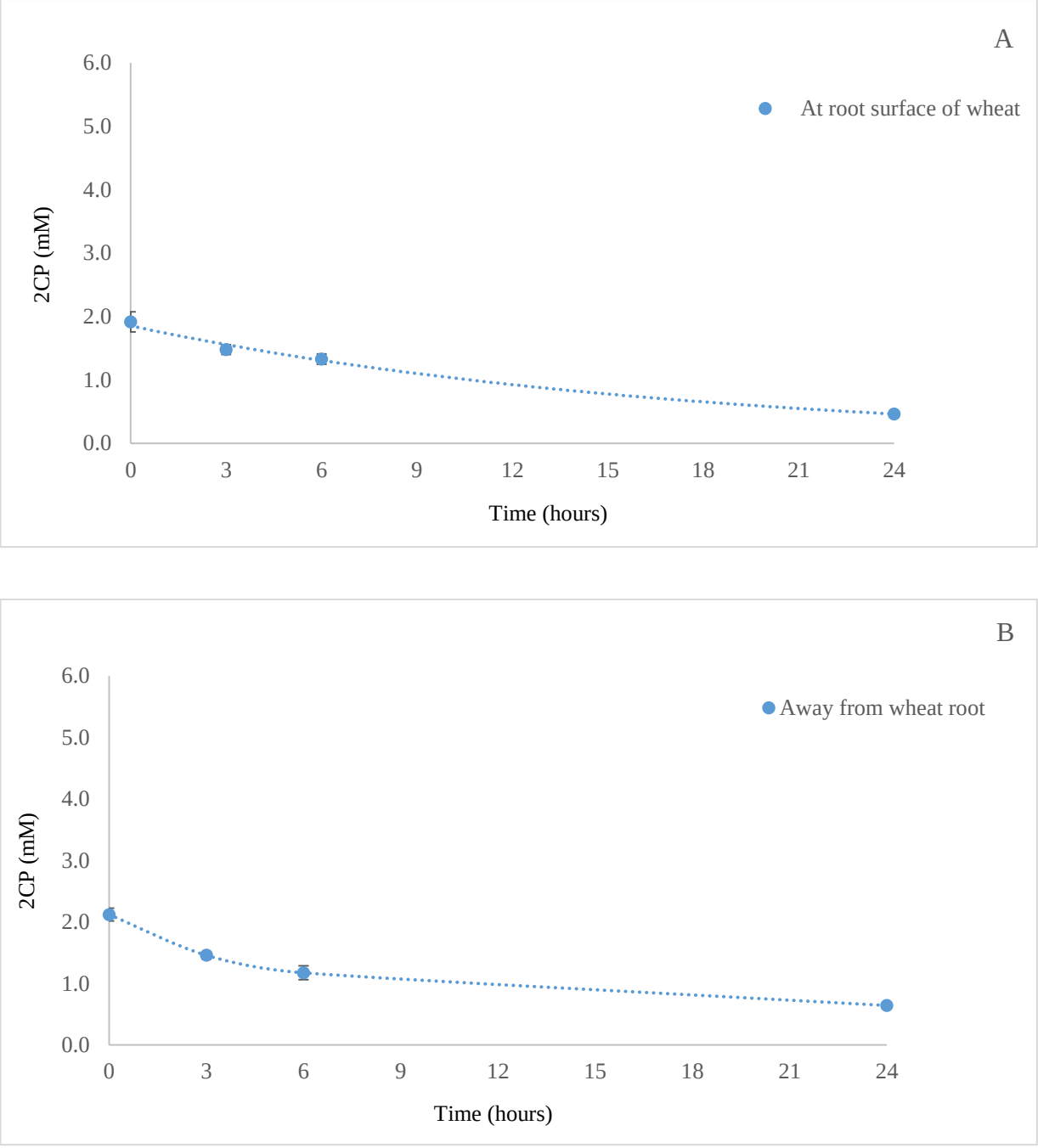


Figure 3.16 Concentration of 2CP in soil extractable moisture was measured over time in presence of wheat plants. Soil samples were collected from around (≤ 1mm) the root (A) and away (>2cm) from the root (B) in the microcosm. The concentration of 2CP in the microcosm at the root of wheat appears to be rapidly reduced in 24 hours (A). It was significantly lower compared to soil sample collected away from the plants roots (B). (Mean ± SE, n =9).

3.4 Discussion

The aim of the experiments described in this chapter was to determine the removal of the selected phenolic compounds that were added to soil in the absence or presence of plants (rye and wheat). Autoclaved and non-autoclaved soil in the absence of plants was studied as controls to evaluate the possible effect microorganism could have on the removal of selected phenolic compounds.

3.4.1 Adsorption

The concentration of the compounds appear to decrease immediately after addition into the soil. This is due the dilution and adsorption that occurs in the soil. This was followed by a degradation processes. Adsorption is one the processes used to remove contaminants such as phenolics from the soil and water (Subramanyam, 2009; Hamza *et al*., 2012; Boufatit *et al*., 2007). According to Gularte *et al*. (2014), the high concentration of phenolic compounds in soil contaminated by accidental spills could be reduced by exploiting of microbes. Bioremediation of these compounds is related with some factors such as adsorption and bioavailability, which depends on natural soil characteristics, pH, and temperature. Many of the bioremediation studies did not take into account the adsorption process, which implies that these results cannot be assumed to be without doubt (Gularte *et al*., 2014).

The adsorption ratio (as defined in this chapter, the higher the ratio the less adsorbed) of most of phenolic compounds was higher in autoclaved soil than non-autoclaved (Table 3.1). This would indicate that sterilisation by autoclaving also effected physical and chemical properties. This has been described by Shaw *et al*. (1999), who revealed that autoclaving increases the concentration of soluble organic carbon that probably comes from organic matter and killed mass and decreased pH in soil. This influenced both adsorption and the ion exchange properties of both soil and solutes. In our study, the soil pH was 6 in non-autoclaved. It was decreased significantly to approximately 5 when soil was autoclaved. This decrease in pH may be due to the release of organic acids (Shaw *et al.,* 1999).

Decreasing soil pH will influence both the (insoluble) soil adsorption surface and the ionisation of the solutes. If we assume that the charges on the solid phase are predominantly anionic (negative) and due to weak acids (carboxylic), decreasing pH will reduce net negative charge and decrease the polarity of the surface. In the case of uncharged solutes, such as phenol and chlorophenol, the influence of this might be expected to increase the adsorptive capacity of the

surface. The data in Table 3.1 indicates that the opposite in the case. Less uncharged solutes are absorbed. One explanation is that the solid charge are cationic (e.g. amino) and that polarity increases with lower pH. However, increasing net fixed positive charge would the expected to remove the organic acid (vanillic and syringic) from solution. The opposite of what in observed.

The decreased soil pH would alter the net charge on the (soluble) anionic phenolic acids (vanillic and syringic). Lower pH will make them less polar. It is unlikely that this would reverse the ion exchange effect.

As this study was carried out in soil, interference by soil matrix is inevitable. The soil characteristics can limit availability of chemical to the root zone. Bioavailability of the compound depends on the physiochemical characteristics of the compound and composition of soil matrix (Flacco *et al*., 2002). Therefore, adsorption of the phenolic compounds into soil after the addition can affect the availability of the said compound to microbes and roots. The table 3.4 shows the percentage of the remaining unadsorpted which was assumed to be available for the degradation in tested soil.

Phenolic Compounds	% Actual quantity of remaining	
	Non-autoclaved soil	Autoclaved soil
Phenol	22*	28
2,4DNP	9	9
3CP	12*	8
2CP	9	NA
Vanillic acid	10*	34
Syringic acid	21*	44
Ethylvanillin	19*	24
DMHAP	17*	25

Table 3.4 Actual quantity of phenolic compounds remaining (%) in soil that was available for degradation process in non-autoclaved and autoclaved soil. However, there was a significant different* in the remaining concentration between autoclaved and non-autoclaved soil ($P < 0.05$) with the exception of 2, 4 DNP.

3.4.2 Degradation

The appearance of fast and slow phases of phenolic degradation is the most curious and unexpected of this project. The fast degradation phase appeared only for some of the phenolic compounds. This must be due to their chemical properties and to the behaviour of the soil they are put in. Masciandaro *et al*. (2013) reported that the fate and behaviour of contaminants is controlled by their physic-chemical properties (e.g. molecular structure, polarity and solubility) in addition to the factors of environment such as pH , water content, temperature and organic matter are influence in the degradation of contaminants.

In case of the slow phase, phenol and some of its selected derivatives (ethylvanillin, DHAMP, vanillic acid and syringic acid) degrade in non-autoclaved soil but at different rates (Table 3.1). In contrast, these compounds degrade at a much slower rate in autoclaved soil. This result indicates that microbes play a role in the (slow phase) degradation of phenol. This is consistent with the work of Baker & Mayfield (1980), who not only showed that phenol was metabolised by microorganisms but that the number of microorganism increase after addition of phenol to the soil.

This suggested that the increased microbial activity was proportionate to the increase in phenol concentration. 2CP was reported to degrade at a rate similar to phenol (Baker & Mayfield, 1980). This is confirmed by this study (Figure 3.4). However, 3CP degrades at a slower rate in both autoclaved and non-autoclaved soil (Table 3.1). This result indicated that the soil microbes are not able to degrade this compound. This is consistent with Baker & Mayfield (1980) who reported that phenol degraded at faster rate compared to 3CP in soil. They claimed that it was able to resist the microorganism degradation or had a toxic effect on these microorganisms. There are abiotic factors such as the (physico- chemical properties of contaminants, pH of soil and temperature that could be influence on the behaviour of contaminants in soil in addition to the availability of suitable microorganisms in soil (Masciandaro *et al*., 2013). The high or low pH would alter microbial activity and effect on the biodegradation of pollutants in soil. The change in pH might have three different effects. (1) Physical effects such as pK of adsorption, anion exchange. Subramanian & Das (2009) report that pH is the most important parameter that effects the removal of phenol by adsorption process. When pH was increased there was a significant reduction in adsorption of phenol in soil. (2) Biochemical effects: enzyme activity is also affected by the changes in pH. (3) Physiological effects: the change in pH may affect

the membrane transport of each species such as (COO^- , COOH) or by the changing of driving force (e.g. Δ pH) which influence the uptake of phenolics.

Vanillic and syringic acid disappeared faster than other compounds. Some studies also propose that phenolic acids added to soil could be depleted rapidly by microbes under favourable environmental conditions after adding them to the soil (Zhou & Wu, 2013). Previous studies have shown that phenolic acid could be utilized as substrates by soil microorganisms (Shafer & Blum 1991; Zhang *et al*., 2010). Syringic acid is one of several allelopathic has been identified in wheat (Wu *et al*., 2000). According to Zhou *et al*. (2014), the effect of allelochemicals on physiological behaviour of microbes is dependent on the concentration and the rate of release from the roots. In cucumber, it is reported to be about 0.01 µmol per day per plant in hydroponic solution. This result in approximately 0.13 µmol / g of syringic acid accumulated in soil (Zhou *et al.*, 2014). The bioactivity range (with respect to microbial activity) is between 0.1 to 1 mmol/ L (Piotrowski *et al.*, 2008).

The results show that the half-life of the three phenols tested (phenol, 2CP and 3CP) in soil in close proximity to the wheat and rye roots was less compared to that away from the roots and the soil without plant. These finding indicate that the plant can effectively promote the removal of phenolic compounds from soil. This may provide one of the *ex-planta* phytoremediation strategies.

According to Alkorta & Garbisu (2001), plants can remove organic compounds in two different ways: 1) direct uptake of organic pollutants and 2) phytoremediation *ex-planta* by root exudation that increase microorganisms growth. Other studies reported that there are three possible ways how phenolic compounds can be rapidly degraded by plants. The plant may uptake the phenol and metabolise in the root tissue (De Araujo *et al*., 2002) or transport it to other parts of plant for metabolism or accumulation (Ucisik & Trapp, 2006). Secondly, plants may indirectly enhance the removal of contaminants by the association with soil microbes; an approach called rhizoremediation (Gonzalez *et al.*, 2013). Root exudates contain a wide range of organic compounds (Jones & Freeman, 2003) and compounds, such as organic acid, alcohols and phenolics, which serve as sources of carbon and energy to enhance the growth of microorganism that are involved in the degradation of organic contaminants by producing enzymes (Alkorta & Garbisu,2001 ; Gozalez *et al* .,2013).

Finally, a plant may secrete some enzymes, which are capable of degrading organic pollutants (De Araujo *et al*., 2002; Alkorta & Garbisu, 2001). Hence, the ability of plants to remove phenol from the soil has been reported by a numbers of authors including Ugrekhelidze (1999), Ucisik &Trapp (2006), Jha *et al*. (2013) and Gonzalez *et al*. (2013). Kang (2014) provides a review of organic contaminant removal with bioremediation and phytoremediation suggesting that there are different mechanisms of phytoremediation such as phytostabilization which mean some plant as immobilize contaminants in the soil through adsorption and accumulation by root. In addition, Susarla *et al*. (2002) reported that phytostabilization and phytotransformation are some of the phytoremediation mechanisms that can be used to remove phenols from the soil.

The experiments demonstrated that wheat and rye are suitable plants for the increasing the degradation of selected phenolic compounds as contaminants in the soil but by which mechanism (uptake or *ex-planta*) remains obscure. The works of others were focused on the removal of phenol by plants in the context of phytoremediation in order to understand the mechanisms of phenol removal form soil and wastewater. Wang *et al.* (2014) used the *Polygonum orientale* plant in his study to remove phenol from wastewater. They reported that the main reasons for the complete depletion of phenol in sterile culture solution were adsorption by the root and metabolism. The disappearance of phenol was attributed slightly to the evaporation of the medium in their study. In addition, De Araujo *et al*. (2002) found absorption and metabolism in their study using hairy root of *Daucus carota* L. However, Gonzalez *et al*., (2013) reported that the plant alone was incapable of degrading completely some contaminants. Therefore, to enhance the phytoremediation processes, plant relationships with bacteria have been suggested and various studies have shown that varied microorganisms have played roles to enhance phenol degradation (Leitao *et al*., 2007; Kurzbaum *et al*., 2010; Afzal *et al.*, 2014; Jiang *et al*., 2014). Although studies in this area have improved, the specific relationship between bacteria and plants in the rhizosphere that are involved in the removal the contaminants is still unknown (Segura & Ramos, 2013). In addition, their study indicated that the selected of plant for phytoremediation is as important as the selection of microbes in bioremediation (Segura & Ramos, 2013). The presented results show that selected plants were capable of removing phenolic compounds from the soil and that rye was faster than wheat to removed phenolic compounds from the soil. This may be due to the difference of numbers and varieties of rhizospheric microorganisms associated with each plant species or it could be due

to different root exudates which contribute to the different reaction between them in the presence of contaminants in soil.

In general, phenolic compounds have varying ranges of stability in the soil and microbes play an important role in the soil to degrade the organic contaminants. Both the rye and wheat plants we tested had the ability to remove the phenolic compounds from the soil. Therefore, to study the biophysical interactions at root/soil interface there is a need to look for the potential of using micro scale technique (section 2.8.2) to sample the rhizosphere at high resolution. The results of this approach are reported in chapters 4 and 5.

Chapter 4: Assessing SiCSA technique for Less-polar solutions (Phenol).

4.1 Introduction

The dynamic fine-scale heterogeneity of the root-soil interface (rhizosphere) provides many challenges for the experimentalist. An example of this is the investigation into the mechanisms that may provide valuable routes to the use of plants to clean up polluted environments; also known as phytoremediation. Root function can vary at the sub-mm level as cells differentiate and mature along the axis (Pritchard *et al*., 1996). Simultaneously polluted soil can be heterogeneous and exhibit gradients at similar resolution. To describe and interpret this situation, we attempted to apply techniques previously developed to study variation between individual plant cells (SiCSA; Single Cell Sampling and Analysis) (Tomos & Leigh, 1999; Tomos & Sharrock, 2001; Tandy *et al*., 2013). Glass microcapillaries mounted on a micromanipulator were used to sample the moisture of soil microcosms in which plants were growing after addition of phenolics. Volumes of 2-5 nanolitres, occupying a soil volume of sub-mm dimensions, were isolated and analysed by capillary electrophoresis (CZE). In order to prevent evaporation of the samples during manipulation, they were placed under water-saturated paraffin oil (Section 2.8.2). From here, they were taken up into the CZE capillary for analysis (section 2.10).

When this technique (micro approach; section 2.8.2) was applied to determine phenol removal from the rhizosphere in the microcosm at high resolution, phenol appeared to disappear rapidly (within 30 minutes) after the addition by spray (2.7.1) to the soil microcosm containing a plant. This was in marked contrast to the macro approach result (Chapter 3) where it took longer to decay in soil, with a half-life of 13 hours (Figure 3. 1) , and 9 hours in soil with wheat plant (Figure 3.11). As shown later in chapter 6, this was not due to rapid removal of phenol due to enzyme activity that was released by plant or microbes. These observation suggested that the "micro approach" (section 2.8.2) using the glass microcapillary and paraffin oil might be leading to a major error. Therefore, an experiment was carried out to compare the disappearance of phenol and 3CP in both macro and micro approach (section 2.8) using different internal standard of L-3, 4-dihydroxy phenylalanine (L-DOPA). In this "macro approach", phenol and 3CP (20 mM) were mixed in the same volume (100 µl) then (100 µl) of

L-DOPA (20 mM) was used as internal standard (Figure 4.1 (A)). In this "micro approach", the same concentration of phenol, 3CP and L-DOPA (as internal standard) was mixed but the volume was 2 nl using a micro capillary (Figure 4.1 (B)). All the samples were analysed using CZE. (Preliminary experiment had shown that L-DOPA was not subject to the same phenomenon as that for phenol (section 4.4, below). The data in Figure 4.1 shows that there was a decrease in phenolic compound concentration over 4 hours in the micro approach, when the sample was in paraffin oil (Plate 2.5 and Figure 4.1B). Meanwhile, no decrease was observed in the concentration by using macro approach (Figure 4.1A). This result indicated that the paraffin oil had effected the phenol and 3CP concentration.

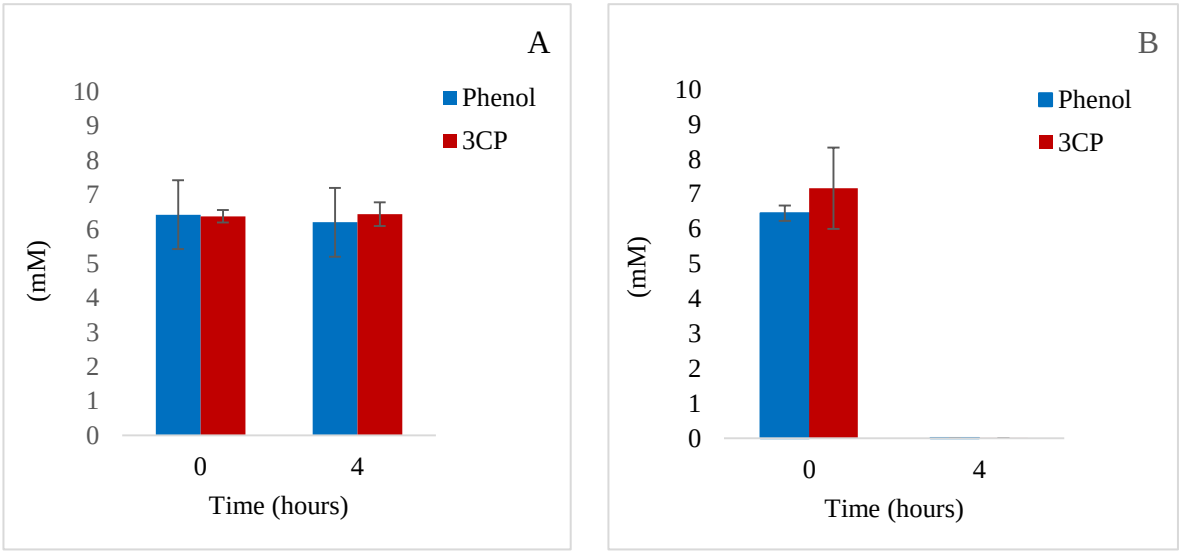


Figure 4.1 Phenol and 3CP concentration after mixing and incubating using L-DOPA internal standard: A) Using macro approach (control). B) Using micro approach. Mean ± SE (n =3).

Three possibilities were investigated. The possibilities that the phenolics were unstable in the borate buffer used, that extracellular enzyme in the extracted moisture were degrading the phenolics in the micro droplet and that the phenolics were being extracted by dissolution into the paraffin oil and lost. This chapter describes the technique development that led to the abandonment of the "micro approach" (section 2.8.2) for the localisation of (less-polar) phenolic compounds. The analytical method (CZE) was used for characterization of phenolic compounds (section 2.10).

During the investigation described, the partition coefficient of phenol was measured using octanol against water. This was compared when paraffin oil and perfluoro compounds were used instead of octanol. The distribution of phenol was different in these experiments. This

lead to the investigation of the replacement of paraffin oil by perfluoro compounds. However, when perfluoro compounds were used to measure the partition coefficient phenol formed three layers. This ternary layer has been used previously for a specific purpose. Albrasi *et al*. (2013) reported that three layered liquid system can form separate phases at specific volumes and temperature by using water, oil and amphiphile. They used these system to grow deposition of nanostructured films of CdSe and CdS as an interfacial deposition scheme.

4.2 Determination of the CZE buffer effect on phenolic compounds

A suggestion was that the compounds might be intrinsically unstable in the borate buffer used for analysis. To test this phenol (20 mM) (100 µl) was mixed with equal amount of 3CP (20 mM) then mixed with (100 µl) of sodium tetraborate buffer (which was used for CZE as describe in section 2.10) over 6 hours to investigate the effect of buffer in these compounds. The data (Figure 4.2) illustrate that phenol and 3CP concentrations appear to be constant over the time.

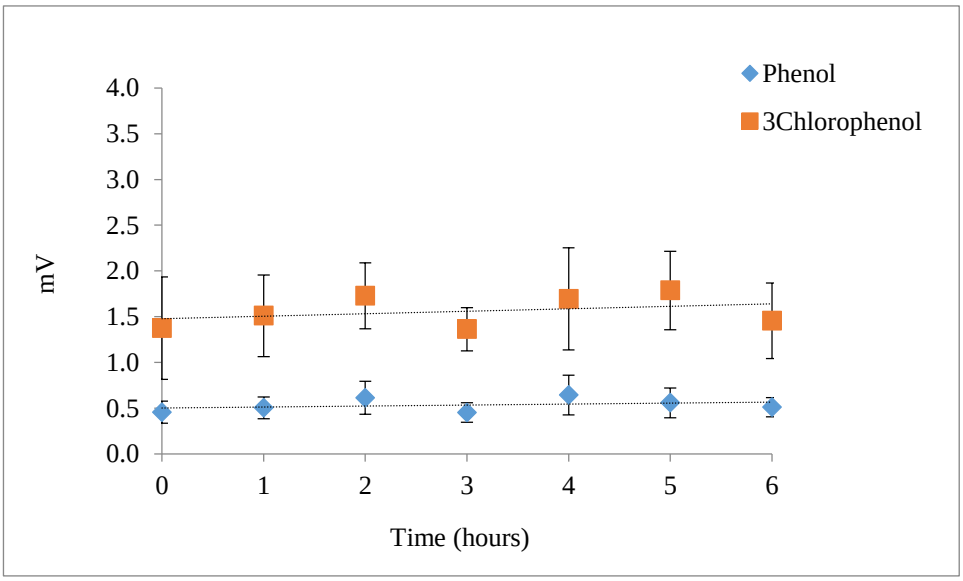


Figure 4.2 The absolute value (not ratioed to an internal standard) of phenol and 3CP after mixing with sodium tetraborate buffer over 6 hours. Mean ± SE (n =3).

Similarly, L-DOPA was mixed with sodium tetraborate buffer (which was used for CZE as described in chapter 2 (section 2.10) over 24 hours to investigate the stability of L-DOPA in the buffer. Only a slight decrease in the concentration was observed over 24 hours (Figure 4.3).

This indicated that L-DOPA was relatively stable and could be used as internal standard to measure phenolic compound concentration.

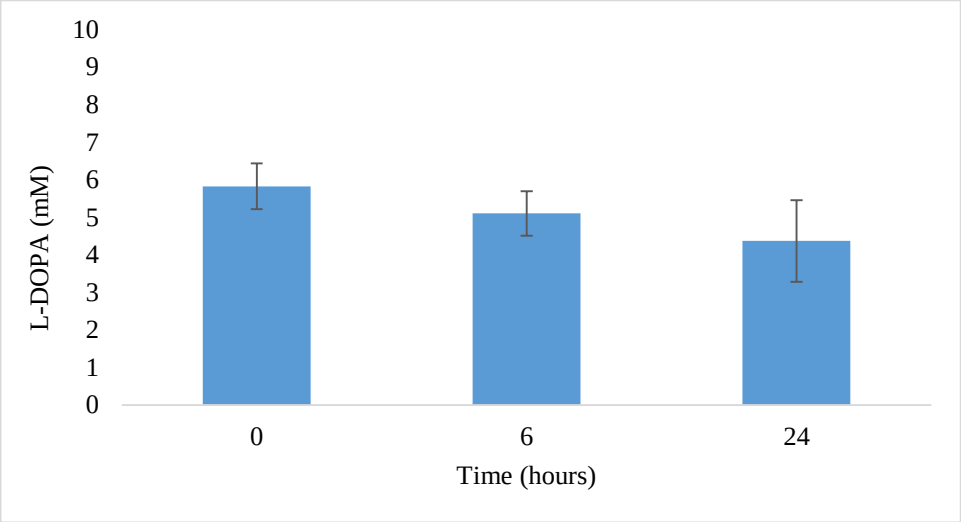


Figure 4.3 L-DOPA concentration after mixing with sodium tetraborate buffer (pH 9.3) over 24 hours.

4.3.1 Using the micro approach to measure effects of enzyme from soil solution on phenol

This experiment was carried out using the micro approach (section 2.8.2) in an attempt to investigate the effect of phenolase from extracted soil solution from wheat microcosm on phenol degradation. A similar experiment was carried out in chapter 6 but using the macro approach. After addition of 20 mM phenol to a wheat microcosm (to induce enzymes (chapter 6)). The micro glass capillary was used to sample the soil solution from the rhizosphere. The micro sample 2 nl of soil solution was mixed with micro sample of (20 mM) phenol as a substrate and kept under paraffin oil (as a step of micro approach (section 2.8.2)). The samples were analysed at regular intervals to determine the concentration of phenol over a period of 3 hours. 3CP was used as an internal standard for the analysis using CZE. The concentration of phenol seemed to be constant (Figure 4.4 A). Also no decrease was observed in a control sample in which was phenol (20 mM) with an equal amount of water instead of soil solution (Figure 4.4 B). The data was consistent with the disappearance of phenol not being due to the phenolase activity from wheat microcosm but due to phenol and 3CP dissolving in paraffin oil at the same rate.

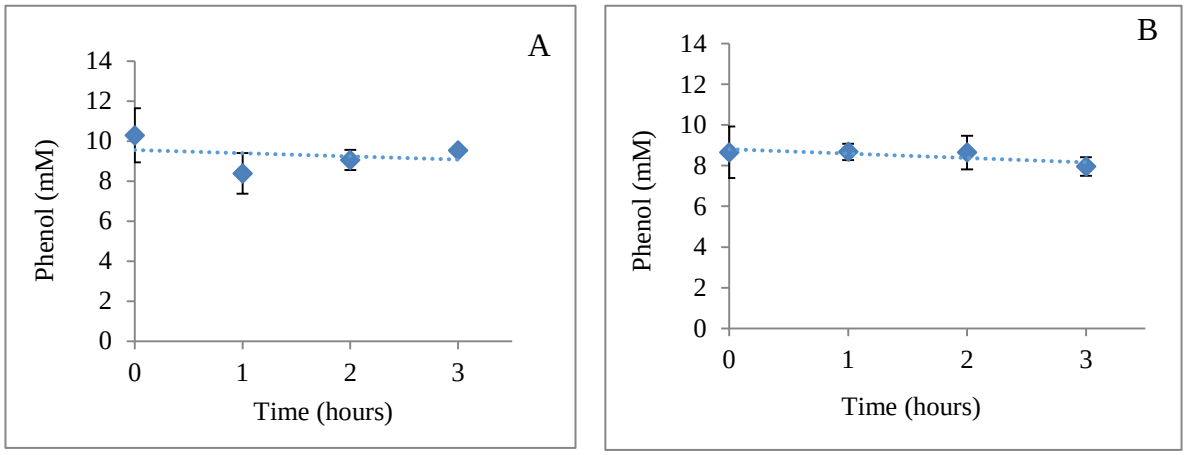


Figure 4.4 The concentration of phenol: A) after mixing (20 mM) phenol with soil solution from wheat microcosm which was treated with 20 mM of phenol to induce enzymes. B) Control sample (20 mM) of phenol with mixed with equal amount of water. (Micro approach).

4.3.2 Using L-DOPA as a substrate to assay phenolase in soil solution

In the literature (e.g. Perucci *et al*, 2000; Uren, 2007) the term phenolase is used to describe enzyme that degrade phenol these are generally phenol oxidases. In this experiment, L-DOPA was used as a substrate instead of phenol to assay the effect of phenolase from soil. L-DOPA is one of the most common substrates that use to assay phenol oxidases activity in root and soil (Bach *et al.*, 2013; Holzapfel *et al.*, 2010).In addition, phenolic compounds in soil are also known to be transformed by oxidative processes catalysed by phenolase produced by the soil microflora and plant roots (Perucci *et al* , 2000; Uren, 2007).

As before, (section 4.3.1 and chapter 6) phenol (20 mM) was added to wheat microcosms (to induce the enzymes). At 30 minutes, soil solution was obtained by sampling the rhizosphere using a microcapillary (Chapter 2 section 2.8.2).This sample of soil solution was subsequently mixed with L-DOPA (10 mM) as substrate. Then it was kept under paraffin oil. The samples were analysed using CZE (section 2.10). The result shows the absolute data of L-DOPA (not ratio to the internal standard). L-DOPA disappeared by 50 % in one hour when it was incubated with soil solution (Figure 4.5). Meanwhile no decrease was observed in control sample, which had L-DOPA with water instead of soil solution. The decrease in L-DOPA is presumably due to the phenolase from wheat microcosm.

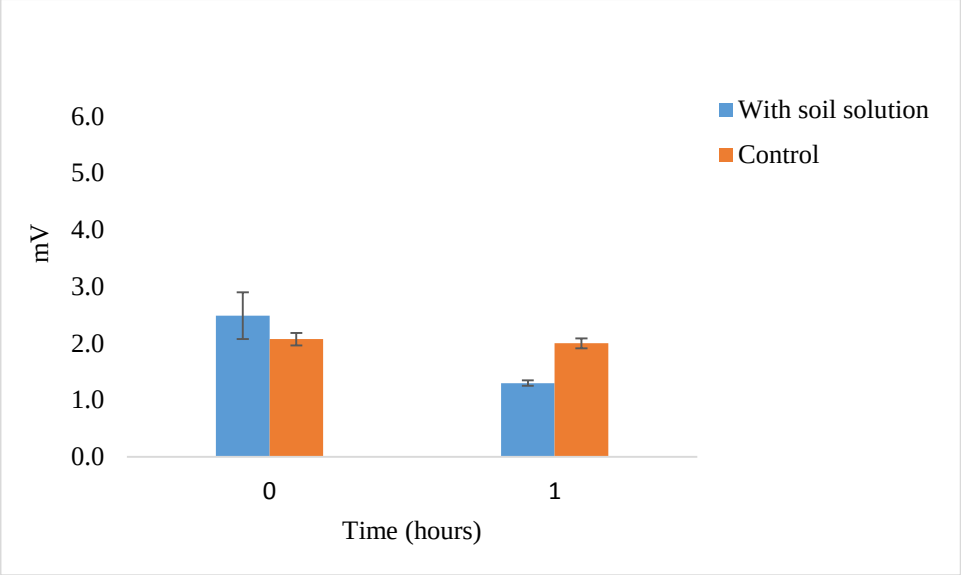


Figure 4.5 L-DOPA (area/ time) ratio after mixing with soil solution from plant microcosm under paraffin oil using micro approach and the control sample (L-DOPA) with equal amount of water.

4.4 Using L-DOPA as internal standard in the micro approach

A key step in this chapter was to show that L-DOPA was not subject to extraction or degradation in the micro-droplet under oil (Figure 4.6). In this experiment, 2 nl micro samples of L-DOPA (10 mM) were kept under paraffin oil. They were measured over one hour without using an internal standard. The demonstration that L-DOPA concentration did not decrease under these circumstances indicates that it is an appropriate material to be used as internal standard to investigate the behaviour of phenol and 3CP under paraffin oil.

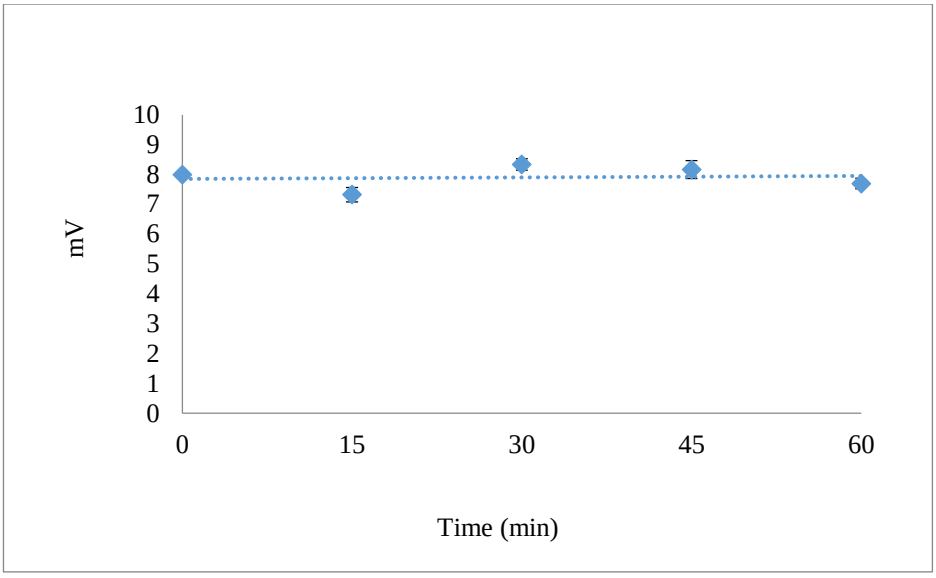


Figure 4.6 The ratio (area/time) of micro droplets (2nl) of L-DOPA (10 mM under paraffin oil over one hour). Mean ± SE (n =3).

4.5 Absorption of phenol by paraffin oil using the micro approach

Using the micro scale (2 nl) approach, the time course of phenol, and some of its derivatives, under paraffin oil was measured using CZE over 60 minutes. L-DOPA was used as internal standard for the other phenol compounds. It was found that phenol and some of its derivatives (3CP, ethylvanillin and 3, 5-dihydroxy-4-hydroxyacetophenone (DMHAP)) apparently dissolved in paraffin within 15 minutes (Figure 4.7 A, B, C, D), while other phenolics such as, syringic acid and vanillic acid were found to remain in the aqueous droplet (Figure 4.8 A, B). In this experiment the time course was determined for phenol (20 mM), 3CP (20 mM) and ethylvanillin (10 mM) separately using equal amounts of L-DOPA as internal standard. However, the other compounds syringic acid (20 mM), vanillic acid (10 mM) and DMHAP (10 mM) were mixed with each other (therefore, the added concentrations were 6.6, 3.3 mM and 3.3 respectively). This solution was mixed with equal amount of L-DOPA (10 mM) as internal standard to determine the time course for these compounds in one experiment.

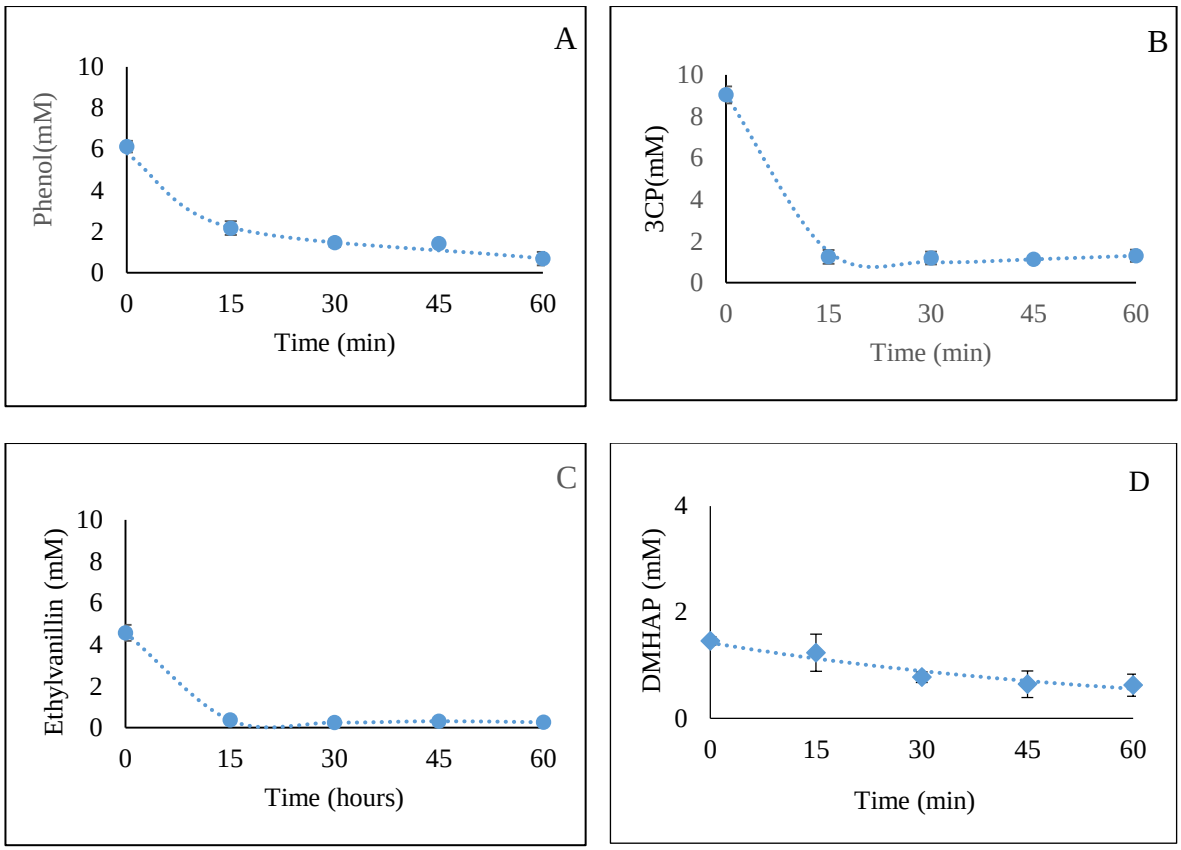


Figure 4.7 Phenolic compounds which dissolve in paraffin oil, (A) phenol (20 mM),(B) 3CP (20 mM). and (C) Ethylvanillin (10 mM). (D) DMHAP (10 mM).(Numbers in brackets refer to the concentration of phenolics in the samples before dilution with internal standard see text section 4.5 for detailes). L-DOPA (10 mM) was used as internal standard in these experiments.

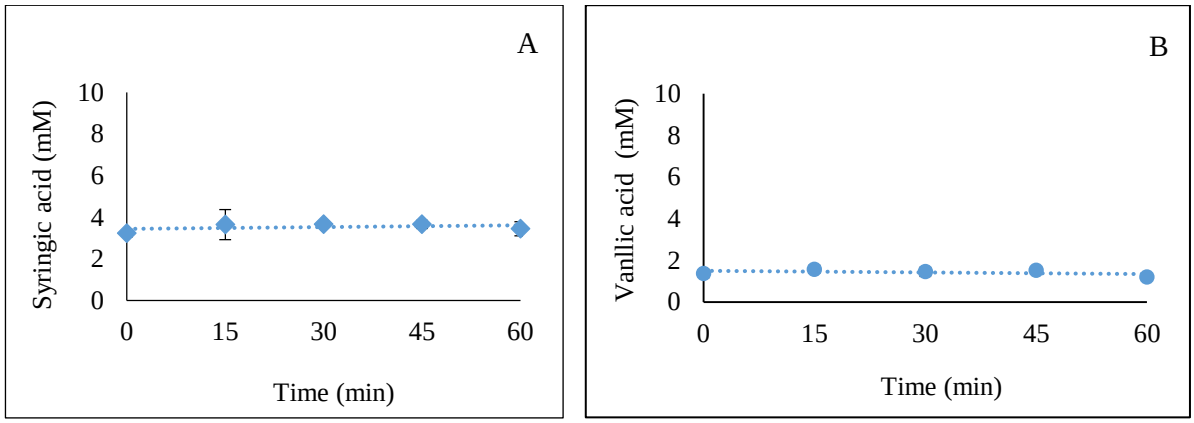


Figure 4.8 Phenolic acids are stable under paraffin oil. (A) Syringic acid (20 mM), (B) Vanillic acid (10 mM). (Numbers in brackets refer to the concentration of phenolics in the samples before dilution with internal standard see text section 4.5 for detailes). L-DOPA (10 mM) was used as internal standard in these experiments.

4.6 Measuring the partition coefficient of phenol

As it appears that phenol and some others of the phenolic compounds are being extracted in the oil, the partition coefficient of phenol was determined using a Shake – flask method by dissolving a small amount of the solute (phenol) in either aqueous (H_2O) or organic phase (octanol). In partition coefficient applications, the octanol-water partition coefficient has been used widely for organic compounds as index to determine the distribution (Sangster, 1989).

4.6.1 Octanol- water partition coefficient K_{OW}

Octanol (0.5 ml) was shaken with (0.5 ml) H_2O and then (100 µl) of 80% phenol solution was added. The mixture was shaken vigorously. After 24 hours the samples that were taken from both separated phases. The top layer (octanol phase) and lower layer (H_2O phase) were analysed using CZE (2.10). L-DOPA (20 mM) was used as internal standard to determined phenol concentration. The partition coefficient of phenol was calculated from phenol concentration in octanol divided by the phenol concentration in water to be 18.4. Partition coefficient is usually expressed as its decadic logarithm. Therefore, the formal partition coefficient of phenol was 1.26. A similar value of 1.46 in the literature (Flocco *et al*., 2002). The partition coefficient from the literature of the others phenolics is shown in table 4.1.There is a good agreement between experimental result that shows L-DOPA did not dissolve substantially in paraffin oil (Figure 4.6) with K_{OW} value of L-DOPA which is -2.39 from literature (Table 4.1). However, the result shows that vanillic and syringic acid appear to remain in the aqueous (nl) droplet (Figure 4.7 A & B) despite the positive K_{OW} values of these compounds that resemble those of the "paraffin soluble" compounds (Table 4.1). A feature that was not taken into account was that K_{OW} of such compounds will be pH sensitive. This might explain the unexpected result.

Phenolic compounds	log (K_{ow})	Reference
Phenol	1.46	Flocco *et al* (2002)
3CP	2.16	GSI
Ethylvanillin	1.82	HMDB
DMHAP	1.67	HMDB
Vanillic acid	1.70	HMDB
Syringic acid	1.55	HMDB
L-DOPA	- 2.39	Sangster (1993)

Table 4.1 Octanol-water partition coefficient of phenolic compounds from the literature.

4.6.2 Paraffin oil

The relationship between the octanol/ H_2O and paraffin/ H_2O partition coefficient was measured since paraffin rather than octanol was used in the project. Water saturated paraffin / water and paraffin oil / water was used to measure phenol partition coefficient using the same method and calculation that were used in octanol / water experiment. The result was 0.95 and 1 for paraffin oil and saturated paraffin oil respectively. Phenol dissolved in octanol twice as much in paraffin. This suggested that paraffin might perform slightly better than would be suggested by the octanol result. But this did not appear to be helpful.

4.6.3 Perfluorooctane and perfluordecalin

Perfluorooctane and perfluordecalin compounds were used in an attempt to find alternative compounds to paraffin that could be used to apply in micro approach (SiCSA) in measurement of the removal of phenol compounds from rhizosphere. The same method (section 4.6.1) and analysis was used to measure the partition coefficient of phenol. It was -1.99 and -2.19 respectively for perfluorooctane and perfluordecalin. These values of phenol partition coefficient were different from previous values using octanol and paraffin oil (Table 4.2).

Organic solvent	log (*K*)
Octanol	1.26
Paraffin	0.95
Water saturated paraffin	1
Perfluorooctane	-1.99
Perfluorodecalin	-2.19

Table 4.2 Partition coefficient of phenol using different solvent for organic phase (against water).

A three-phase system formed when perfluorooctane and perfluorodecalin were used in this experiment. From the presence of an intense brown colour at the interface, it would appear that phenol was concentrated in this interface (Plate 4.1). The concentration of phenol at the interface, water and perfluoro layers for both compounds from the total phenol added (912 μMoles) is shown in Table 4.3. A three-phase system is used to promote intimate mixing of oil and water and maximise the rate and extent of chemical interaction (Strey, 1996; Dong & Hao, 2010). There are no previous reports of ternary liquid formation in the case of water-phenol-perfluoro. However, water, amphiphile, oil systems are known to form three layers under right condition of concentration and temperature (Strey, 1996; Dong & Hao, 2010). Regarding to SiCSA technique, this interfacial layer might provide a suitable sample for the CZE (or other) analysis technique, under those conditions when paraffin oil is unsuitable.

Compound	Layer	Phenol concentration (µMoles)
Perfluorooctane	Water	250
	Perfluorooctane	2.50
	Interface	659.50
Perfluorodecalin	Water	271.25
	Perfluorodecalin	1.75
	Interface	639

Table 4.3 The concentration of phenol in three phases (interface, water and organic phase) of Perfluorooctane and perfluordecalin.

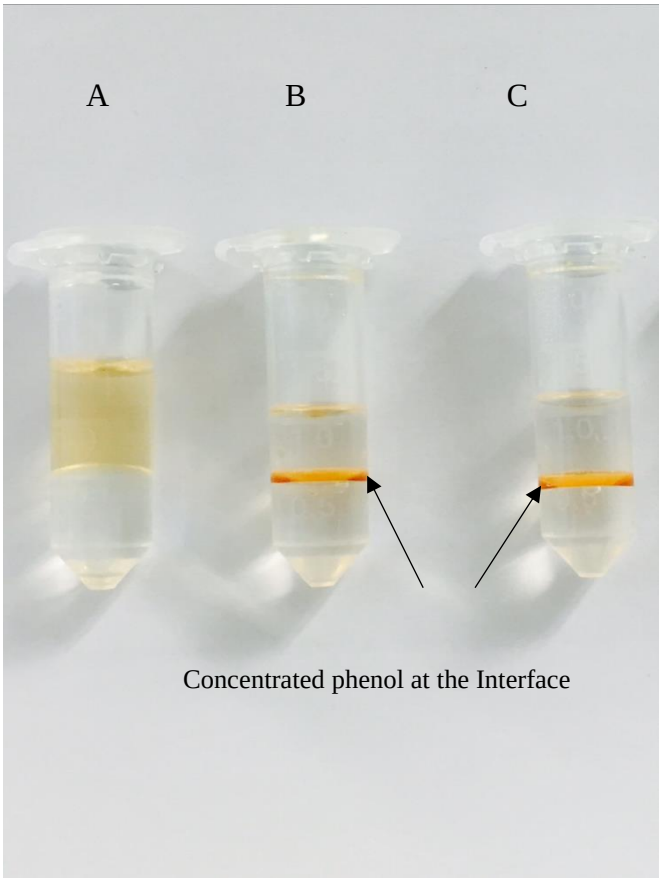


Plate 4.1 Different types of layers formed when different proportions of chemicals are used (A) Mixture of octanol / water; (B) mixture of perfluorooctane / water; (C) mixture of perfluorodecalin / water).

4.7 Discussion

Organic pollutants of lower polarity, such as phenol, 3-chlorophenol offer different technical challenges when the fine-scale SiCSA technique are used. Classical SiCSA techniques were less suitable for these compounds as they readily dissolved under paraffin oil. Syringic acid and vanillic acid, however, were more suitable using these techniques presumably because they are more polar (despite the available value of (K_{ow}) (Table 4.1) that appears to be inconsistent with this result). Therefore, the micro-approach (SiCSA) was used to measure syringic acid presence in the plant's rhizosphere (Chapter 5). L-DOPA was found to be suitable for use as internal standard in micro approach experiments under paraffin oil.

The three phases formed when perfluoro compounds were used may possibly lead to their use instead of paraffin oil to store the nl sample using micro approach. Phenol could be concentrated in the interface of micro droplet sample and this may facilitate drawing of the sample by CZE capillary for analysis. This was not attempted in this study (as this came too late in the period). Therefore, high resolution work continued for polar phenolic such as syringic acid as described in the next chapter.

Chapter 5: Syringic acid behaviour in soil with a plant using ultra-fine spatial scale sampling

5.1 Introduction

In chapter 3, the behaviour of phenolic compounds in soil samples taken from close and far from a root system were compared, and also compared with control soil samples. Those measurements were made at relatively low resolution. For this chapter, similar measurements were made at far higher resolution and different distance from the root. (The difficulties of this approach for phenol and other non-polar phenoilcs were dealt with in chapter 4).

Given the heterogeneity of soil, measuring bulk parameters only produces averaged information, potentially hiding important details of process mechanisms. This resolution issue were overcome by sampling soil at fine spatial resolution using a modified Single Cell Sampling and Analysis approach SiCSA (Tomos & Sharrock, 2001). SiCSA has recently been applied to soil sampling by Tandy *et al*. (2013). This technique involves the use of microcapillaries to sample ~ 2 nanolitres volumes of soil solution and the analysis of their contents. It proved possible to use this approach to measure phenolic acid presence in plant rhizosphere at very fine spatial resolution. We carried out a procedure using SiCSA to measure the disappearance of syringic acid from soil at different distances from the surfaces of wheat roots growing in the same microcosm system as described in chapter 3.

5.2 Half- life of syringic acid in soil at the root surface of wheat plants.

Microcosms were sprayed with 20 mM syringic acid as described in chapter 2 (section 2.7.1). This concentration (20 mM) was chosen to be constant with phenol and chlorophenols to compare the behaviour in soil at the same concentration. Micro samples (2 nl) of extractable soil moisture were obtained as described in chapter 2 (section 2.8.2). Syringic acid concentration was measured by CZE (section 2.10). Initial concentration decreased rapidly from zero time. As described in chapter 3 (section 3.2), there is a rapid dilution of the 20 mM applied by the soil moisture present in the microcosm. This was predicted to give a "zero time" concentration of 11 mM. In chapter 3, this was followed by an apparent rapid adsorption. A similar effect appears to have occurred here (Figure 5.1), where the initial values for syringic acid were 6.1 and 7 mM. Figure 5.1 suggests that the root has an effect on the adsorption (no significant difference between the initial concentration of syringic acid in soil and the concentration at root surface $P > 0.05$). In this chapter, the concentration of syringic acid at zero time was higher in the control (soil only) than the control in (Chapter 3) using macro approach. Subsequently the results showed syringic acid disappeared rapidly from the soil. Both the $t_{½}$ of syringic acid at the surface of the root and the $t_{½}$ in the control without a plant were under 2 hours (Figure 5.1). Although there was a significant difference between their $t_{½}$ ($P < 0.05$).

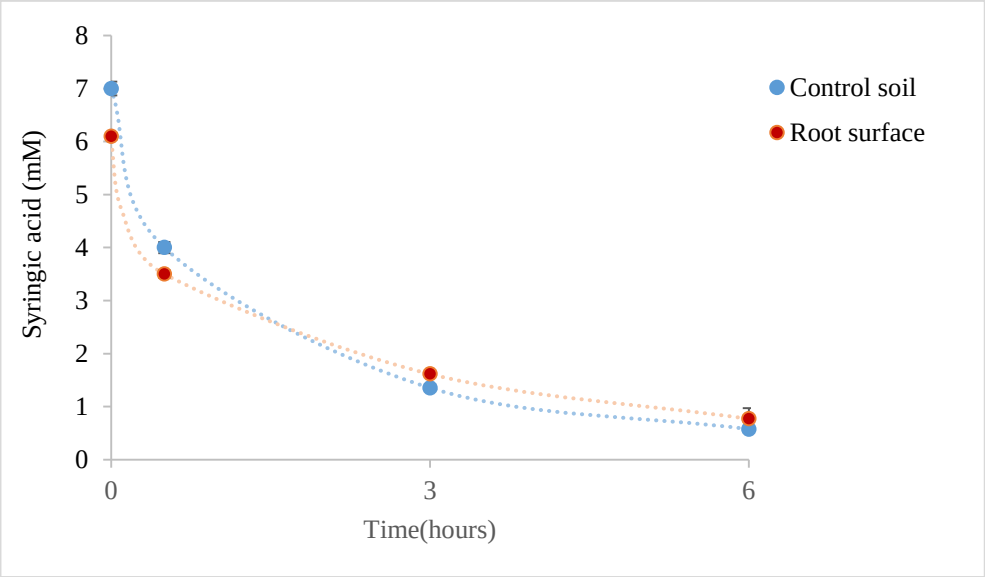


Figure 5.1 Half-life of syringic acid at the root surface in wheat microcosm and in control soil microcosm without a plant after the addition of (20 mM). A microcapillary was used to sample (~2 nl) volumes of soil solution. Data shown are mean values ± SE (n =9).

5.3 Concentration of syringic acid at discrete distances from the root.

The concentration of syringic acid was determined at discrete distances away from the root at 30 minutes after spraying the soil with a 20 mM solution in order to determine the syringic acid dynamic in the rhizosphere of wheat seedling. Approximately 2 nl volume of sample was taken at the root surface then from 2 and 4 cm away from the root (Figure 5.2). A gradient of syringic acid concentration away from the root was observed (Figure 5.2) indicating that the root is influencing rhizosphere concentrations. To improve resolution, in a second experiment, the same amount of syringic acid was sprayed but samples were taken at 1, 2, and 3 mm from the root surface (Figure 5.3). One mm is the minimum measurable distance because 2 nl soil moisture occupies approximately $1mm^3$ of ventilated soil at 10 % water content (Tandy *et al*., 2013). The concentration decreases gradually away from the surface. At the distance of 2 mm away from the root, the concentration gets to 2.2 mM and at 4 cm it gets to 1.8 mM. This is consistent with Figure 5.2 and allow the two to be superimposed.

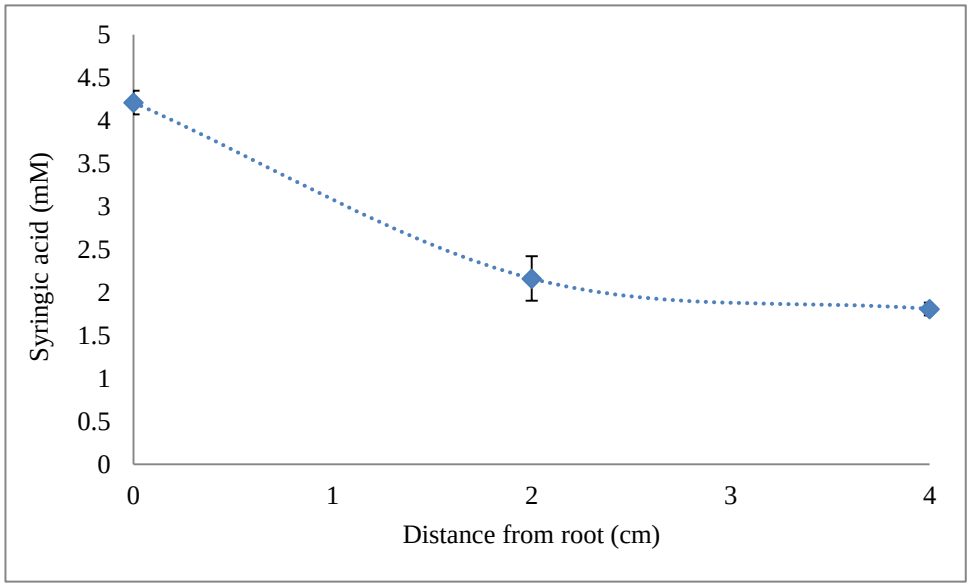


Figure 5.2 Syringic acid concentration gradient measured starting from the root surface, 2 cm and 4 cm distance from wheat root, shows a gradual decline in concentration away from the root (taken at 30 minutes). Mean ±SE (n=3).

One explanation for this radial gradient of syringic acid is a "sweep away" effect due to transpiration drawing soil solution toward the root surface (from where the solutes are not taken up by the root). In order to determine the influence of transpiration on the syringic acid distribution in soil, the same experiment was repeated after removal of the leaf. (This should reduce transpiration to close to zero). There was a significant difference in syringic acid concentration at the root surface between plant with leaf and plant without leaf ($P < 0.05$) (Figure 5.3).The results show that the gradient was partially abolished by the removal of the leaves. This suggests a role of transpiration in the formation of the gradient.

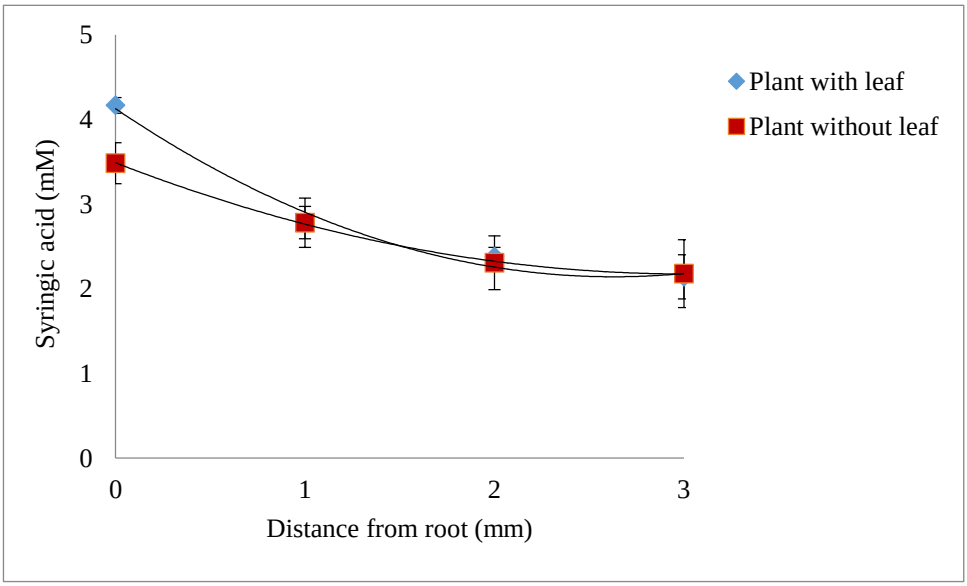


Figure 5.3 The syringic acid gradient generated by the root is partially reduced by removal of the leaves (decreased transpiration). Illustration at 30 minutes post spray. There is significant difference at $P < 0.05$ of the concentration at the root surface (0 mm). Mean ±SE (n=3).

5.4 Discussion

Monitoring the distribution of syringic acid at various distance from root surface after 30 minutes of introduction to soil, showed a higher concentration at the root surface. There was dramatic declination at 1 mm, 2 mm from the root. This higher concentration at the surface of root could be attributed to different reasons. It is possible that wheat roots secreted syringic acid into soil. But we know from the control samples of root (without any spray) that no syringic acid was detected in the root or in the rhizosphere. On the other hand, syringic acid movement to the root surface could also be due to the bulk flow driven by hydrostatic pressure and transpiration force which is the most important for the movement of water and dissolved chemicals into the xylem. Meanwhile, syringic acid will be driven in the opposite direction by diffusion once the gradients observed are established (Nobel, 1999). These two processes could be expected to set up and maintain a steady state of an unstirred layer. Data from the experiment carried out by cutting the leaf in order to determine the effect of transpiration, showed a significant difference between the concentration gradient after leaf removal. The gradient was most likely due to transpiration flow. Thus, from this result, transpiration stream partially attributes to above syringic acid behaviour. The other effect of the plant root might be to produce antibiotic that influences on the rhizosphere microbes activity. No evidence was seen of syringic acid being degraded by enzyme from the root. In contrast to chapter 3 where away from the root depletion of phenolic compounds was found to be slower than at root surface which referred to secretion of enzyme in the rhizosphere as one of possible effect of roots.

Syringic acid removal from non-autoclaved soil has been shown to be faster than for autoclaved (Chapter 3) indicting a microbial role in the fast disappearance of syringic acid. However, despite the rapid disappearance of syringic acid from the soil both with a plant and in the absence of plant (both under 2 hours), the root may still play role in the rapid disappearance syringic acid. The rhizosphere, the region of soil around the root, was influenced by exudation, root uptake and activities of associated soil microbes. There also might be a competition between plant and microbes for the nutrients. According to the results to be shown in (Chapter 7) substantial quantities of syringic acid were detected in wheat roots after 30 minutes of addition to soil. This indicates a root effect on the disappeared of syringic acid from soil by the uptake. Syringic acid may be turned over very rapidly by microbes and plant roots. Previous studies reported that phenolic compounds rabidly disappeared from soil solution due to microbes utilization, roots uptake or by the adsorption by soil partials (Zhou *et al*, 2014).

Chapter 6: *Ex-planta* removal of phenol by release of plant enzyme

6.1 Introduction

Plants have several mechanisms to remove contaminants, such as phenols, from soil, ground water and air by means of absorption and accumulation of the contaminant in plant tissue (Rao *et al* .,2010). Plants also have other mechanisms to remove contaminants. Some plants can secrete degrading enzymes into the rhizosphere or excrete root exudates which stimulate microorganisms to degrade organic pollutants (Rao *et al.*, 2010; Susarla, 2002).

Phenol and its derivatives, when found as contaminants, can be removed by a number of plant and fungal oxidases, such as laccase and tyrosinase, in order to reduce their toxicity (Torres *et al*., 2003; Wolfe & Hoehamer, 2003). Phenolases are reported to be some of the enzymes that are present in rhizodeposits (Hirsch *et al*., 2013) and released by plant roots (Uren, 2007). Sinsabaugh (2010) reported that, for different objectives, plants and microorganisms produce intracellular and extracellular phenol oxidases. Phenol oxidases are used by plants to synthesize compounds such as lignin; whereas microorganisms use phenol oxidases to synthesize protective compounds such as melanin. Even though they play different roles and have different functions, they enter the environment by secretion or cell decay.

Several studies have reported enzymes released by different roots of plants for the purpose of degradation of phenols. Gramms *et al*. (1999) identified enzyme such as peroxidase and laccase exuded by root of 12 terrestrial plant species into sterile and non-sterile soil. These enzymes also might be detected on root surfaces of some plants such as wheat, cotton and tomato. They suggested that peroxidase from plant tissue and root surface detoxify organic contaminant such as phenolic compounds. However, they also reported that secretion of enzyme was enhanced in fertilized, dying and water-stressed plants. Jha *et al*. (2013) studied the removal of phenol by hairy root of *Helianthus annuus*. They reported that peroxidase enzyme activity was induced 24 hours after addition of phenol to the root compared with control of only phenol solution. In addition, they found the rate of phenol removal to be enhanced after the addition of H_2O_2 to the assay mixture. Gonzalez *et al*. (2006) demonstrated in their study, the ability of tomato

hairy root to be source of enzyme which removes phenol from the culture within one hour. (Therefore, a short induction was used in this project).

Single cell sampling and analysis SiCSA (Tandy *et al.,* 2013) was used to sample the soil solutions from the microcosm after the addition of phenol using a micro-capillary at a very fine resolution (~2nl) (chapter 2 section 2.8.2). Phenol was found to disappear rapidly from the soil. Therefore, due to the apparent fast disappearance of phenol using micro scale the experiments, this chapter was designed to investigate *ex-planta* phenol degradation in soil and the effect of induced and non-induced activity of enzyme. Removal of phenol by phenolase released by wheat plants or induced microbial activity was estimated by measuring the change of concentration of phenol in a standard solution catalysed by soil solution that was obtained by centrifugation from a wheat microcosm (section 2.8.1). The disappearance of phenols was monitored using three different methods. These were Capillary Zone Electrophoreses (CZE) (section 2.10), a microtiter plate) (section 2.13.1) and a conventional spectrophotometer method (section 2.13.2). The enzyme activity was estimated by the change in the absorbance of the substrate in the presence of soil solution.

6.2 Determination of phenol degradation by non-induced enzyme in soil solution using CZE

In order to determine if the rapid phenol disappearance from the microcosm occurred directly due to soluble enzyme that had been released by the wheat plant or by enzymes that were already present in the soil before addition of phenol (as non - induced enzyme), soil solution was extracted from microcosms by centrifugation then added to phenol solution. CZE was used to measure phenol concentration (chapter 2, section 2.10) using 3chlorophenol (3CP) as internal standard. The assay mixture contained a 100 µl sample of soil solution from the microcosm, with or without plant, sampled into separate 1.5 ml Eppendorf tubes. Phenol solution 20 mM (100 µl) was added into each of these solutions and mixed. The mixture was incubated for 30 minutes at room temperature. The blank sample contained water (100 µl) with (100 µl) of 20 mM phenol. Concentration of phenol was measured at hourly intervals for 4 hours. All the samples of soil solution from microcosms without a plant and microcosms with a plant showed no degradation of phenol (Figure 6.1), suggesting that the rapid disappearance of phenol in soil was not due to non-induced activity of water soluble enzyme released by the plant or microbes in soil.

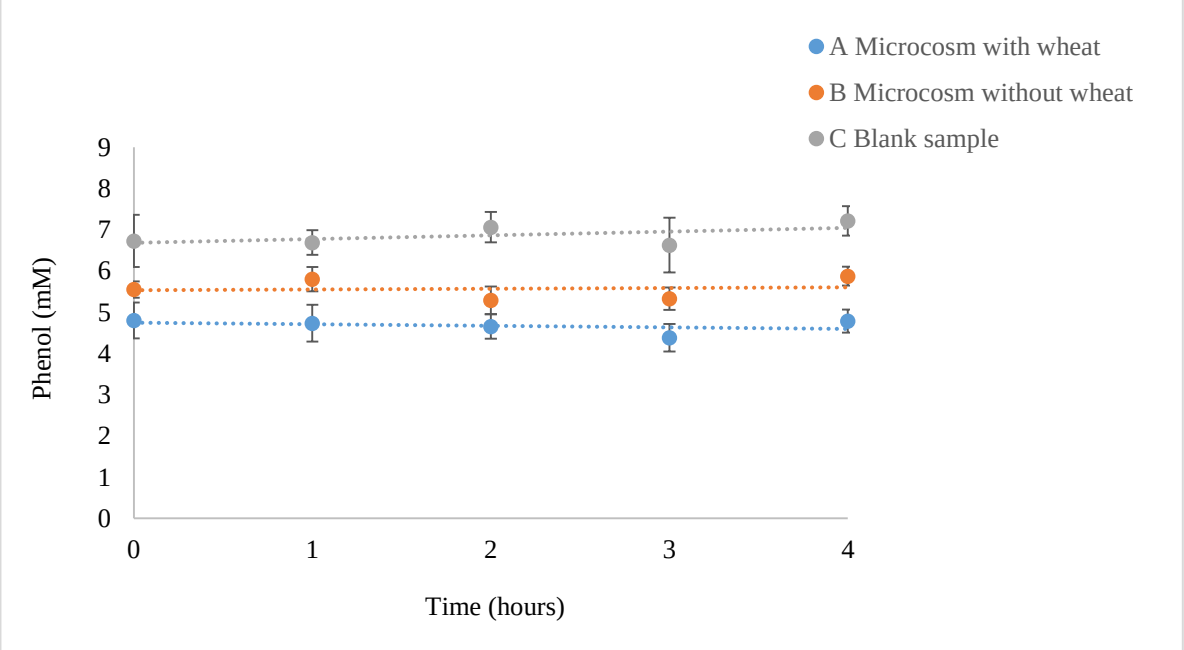


Figure 6.1 Degradation of phenol by non-induced enzyme in soil using CZE was determine by measuring phenol concentration at hourly intervals for 4 hours after mixing with soil solution.
A) Soil solution from microcosms with wheat (100 µl) + (100 µl) of 20 mM phenol.
B) Soil solution from microcosms without wheat (100 µl) + (100 µl) of 20 mM phenol.
C) Blank sample (100 µl) H_2O+ (100 µl) of 20 mM phenol.
All the experiments were set up in triplicate and the observations were averaged and SE calculated.

6.3 Determination of phenol degradation by induced enzyme in soil solution using microtiter plate method

The effect of phenolase on the rapid disappearance of phenol was investigated by measuring phenol concentration using a microtiter plate UV-spectrophotometer method (section 2.13.1) using a wavelength of 250 nm. A set of standard sample solutions with a phenol concentration ranging from 0.5 to 10 mM of phenol (200 µl) was used to generate a calibration curve of absorbance values (Figure 6.2).

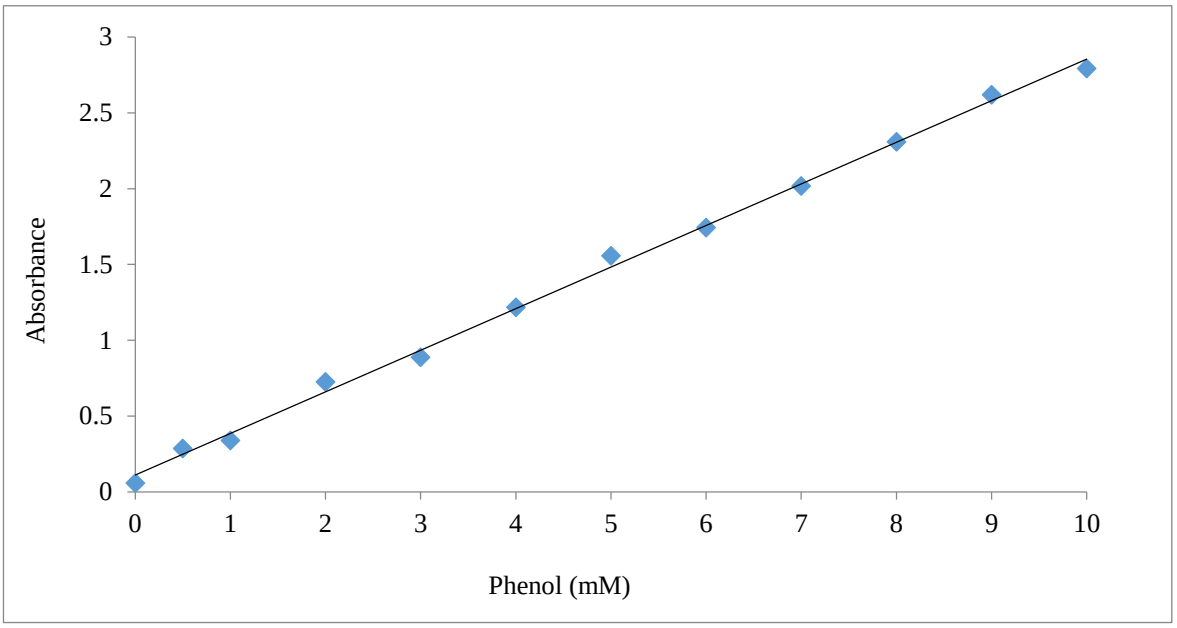


Figure 6.2 Standard curve drawn to measure concentration of phenol against absorption at 250 nm using plastic microtiter plate.

6.3.1 Measuring the disappearance of phenol incubated with soil solution from a microcosm with a plant

The effect of soil solution as a source of enzyme on degradation of phenol concentration was determined using a microcosm with a wheat plant. In this experiment microcosm was sprayed with 20 mM phenol to induce activity of enzyme. After 30 minutes of incubation at room temperature, a 100 µl sample of soil solution was obtained by centrifugation (from the microcosm with plant after spraying). It was mixed with 100 µl of 5 mM phenol as substrate in a micro-plate. Phenol concentrations were calculated from phenol standard curve (Figure

6.2). The assay mixture included the phenol used to induce enzyme activity in the soil solution that was centrifuged from the microcosm after 30 minutes of spraying. In the final assay, phenol concentration at zero time was approximately 5 mM. This was estimated as 2.5 mM diluted from soil solution concentration at 30 minutes of addition (chapter 3 section 3.3) and 2.5 mM from the second addition of 5 mM phenol as substrate with exception of blank sample). A decrease of 8.5% in phenol concentration was observed over 6 hours (Figure 6.3 D). This corresponds to a phenolase activity of 0.14 mMoles/L.hr in soil solution of wheat microcosm. However, in the blank samples (which had 5 mM of phenol (100 µl) mixed with (100 µl) water) a decrease of 4.1% phenol concentration also was observed (Figure 6.3A). However, the percentage of the decrease of phenol over 6 hours was significantly higher in the presence of plant compared to the blank samples ($P < 0.05$).

6.3.2 Measuring the disappearance of phenol incubated with soil solution from a microcosm without plant

The same experiment was repeated but with soil solution from microcosms without plants. These were obtained by centrifugation (as described in section (2.8.1)) to compare the change of phenol concentration by microbes without plant with the data of microcosm with wheat plant (6.3.1) over 6 hours. The percentage yield will depend on the centrifugation force. It will be dominated by the larger pores that are more easily drained (Edmunds & Bath, 1976 ; Yokoyama *et al*., 2011). It is assumed that all pores have similar phenolic concentration regardless of size). The result showed a reduction of 6.7% phenol in 6 hours (Figure 6.3B) and the corresponding phenolase activity was 0.09 mMoles/L.hr in soil solution of microcosm. Here the percentage of phenol decrease over 6 hours was significantly lower than the decrease of phenol by soil solution from wheat plant microcosm ($P < 0.05$).

6.3.3 Measuring the disappearance of phenol incubated with soil solution from an autoclaved soil without a plant

Soil without a plant was autoclaved to investigate if microbes have any role in the decrease of phenol concentration in soil microcosm. In addition, the autoclaving process might attribute to increase enzyme activity due to release from microbes. Soil solution (100 µl) was obtained by centrifugation from soil to mix with (100 µl) of 5 mM phenol. The results showed a decrease

in phenol concentration, which was 5% over 6 hours (Figure 6.3C) and the corresponding phenolase activity was 0.06 mMoles/L.hr in soil solution of microcosm. The percentage of phenol decrease was lower than non-autoclaved soil ($P < 0.05$). The autoclaving process may have effected enzyme activity.

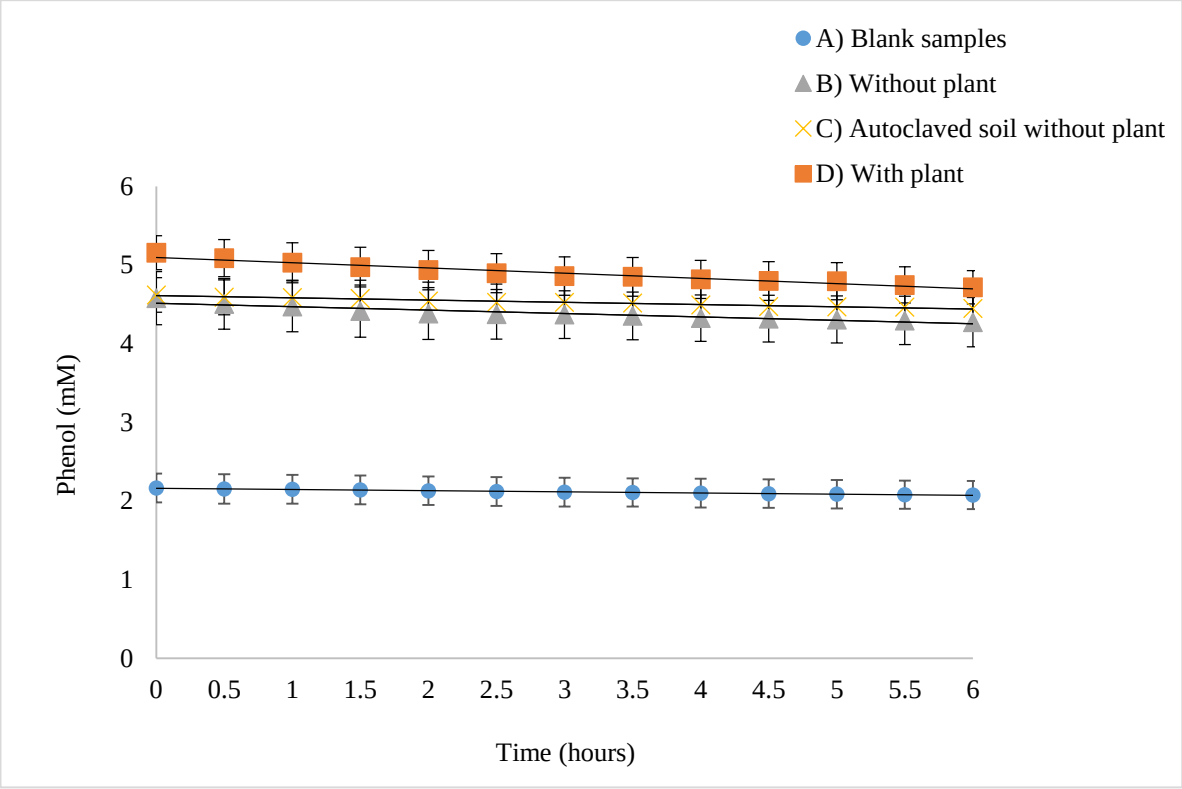


Figure 6.3 Degradation of phenol by induced enzyme in soil was determine using microtiter plate by measuring phenol concentration at hourly intervals for 6 hours after mixing with soil solution.
A) Blank sample (100 µl) H_2O+ (100 µl) of 5 mM phenol.
B) Soil solution from microcosms without plant (100 µl) + (100 µl) of 5 mM phenol.
C) Soil solution from autoclaved soil without plant (100 µl) + (100 µl) of 5 mM phenol.
D) Soil solution from microcosms with plant (100 µl) + (100 µl) of 5 mM phenol.
All the experiments were set up in replicates of nine and the observations were taken as average plus (SE).

6.4 Determination of phenol degradation by induced enzyme in soil solution using a spectrophotometer method

A spectrophotometer using a glass (3 ml) cuvette at 250 nm wavelength was used to estimate the phenol removal due to enzyme activity over 6 hours. This was attempted as phenol concentration in blank sample might have been undergoing adsorption when using the plastic microtiter plate. A standard curve was plotted for absorption at 250 nm wavelength against

concentration ranging from 1 to 10 mM of phenol (3 ml). This standard curve was used to determine the concentration of phenol assay (Figure 6.4).

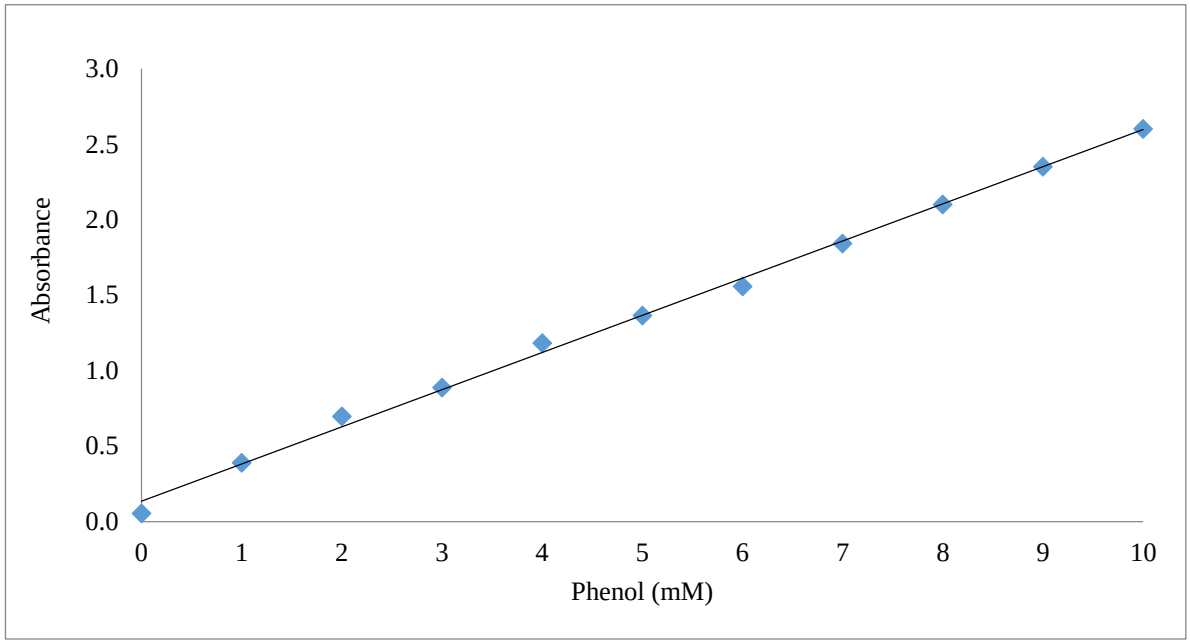


Figure 6.4 Standard curve drawn to measure concentration of phenol against absorption at 250 nm using 3 ml glass spectrophotometer cuvette.

6.4.1 Measuring the disappearance of phenol incubated with soil solution from microcosms with wheat plants

The soil from wheat microcosm was centrifuged 30 minutes after spraying with 20 mM phenol to induce possible enzyme activity then mixed with phenol 5 mM (1.5 ml) in order to determine the change in phenol concentration over 6 hours. The final mixture in the glass cuvette (3 ml) size included (0.5 ml of soil solution, 1.5 ml phenol and 1 ml water). The result shows that there was a decrease 12% in phenol concentration over 6 hours (Figure 6.5B). The corresponding phenolase activity was 0.2 mMoles/L.hr in soil of wheat microcosm. In the blank sample which had 5 mM (1.5 ml) phenol with (1.5 ml) water, no decrease in concentration was observed over 6 hours (Figure 6.5A).

6.4.2 Measuring the disappearance of phenol incubated with soil solution from microcosms without a plant

Soil solution was extracted by centrifugation from the microcosm without a plant (1.5 ml) and added to (1.5 ml) 5 mM of phenol in order to determine the concentration over 6 hours. The data shows a decrease of 7.4 % (Figure 6.5C) and the corresponding phenolase activity was 0.18 mMoles/L.hr in soil solution of microcosm.

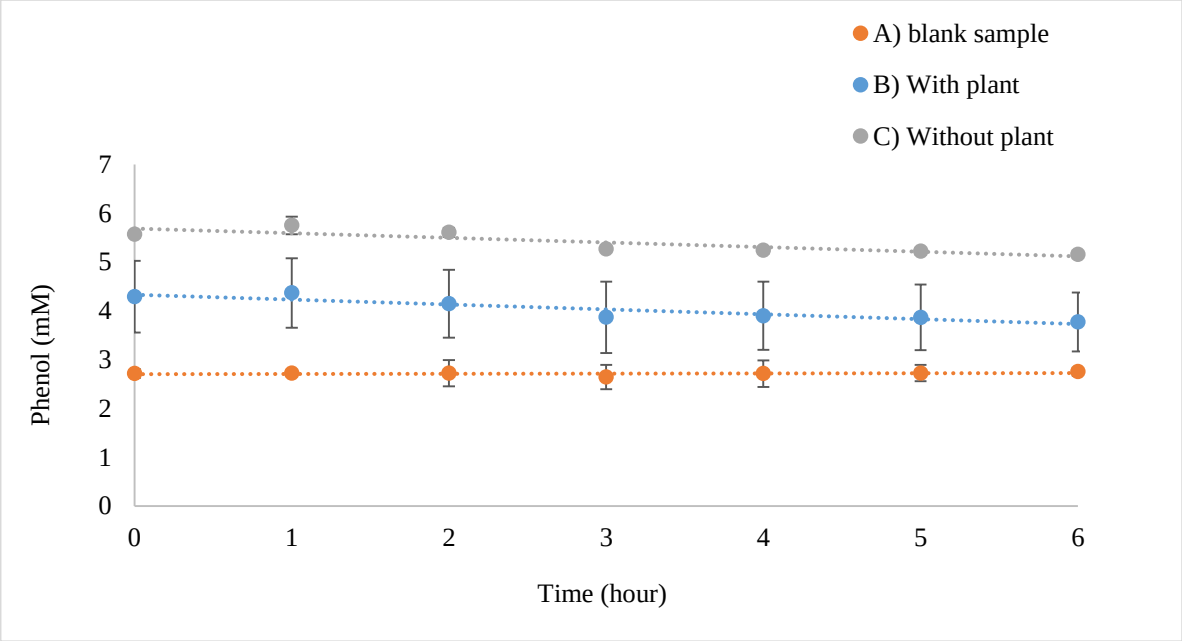


Figure 6.5 Degradation of phenol by induced enzyme activity in soil solution using a spectrophotometer after spraying of wheat microcosm with phenol.
(A) Blank samples (1.5 ml) H_2O+ (1.5 ml) of 5 mM phenol.
(B) Soil solution from microcosm with plant (1.5 ml) + (1.5 ml) of 5 mM phenol.
(C) Soil solution from microcosm without plant (1.5 ml) + (1.5 ml) of 5 mM phenol.

	Microtiter plate		Spectrophotometer	
	Phenol concentration decrease %	Phenolase activity (mMoles/L.hr)	Phenol concentration decrease %	Phenolase activity (mMoles/L.hr)
Microcosm with plant	8.5 *	0.14	12.0*	0.20
Microcosm without plant	6.7 *	0.09	7.4*	0.18
Autoclaved samples	5.1*	0.06	NA	NA
Blank samples	4.1*	-	0.00	0.00

Table 6.1 Comparison of the percentage of phenol degradation measured using Microtiter Plate and Spectrophotometer between the microcosm with plant, without plant, autoclaved and blank samples. The one-way ANOVA on this data revealed a significant difference * between the percentages of phenol decrease over 6 hours using different treatments (microcosm with plant, without plant, autoclaved soil and blank samples). A value of $P < 0.05$ was taken as significant.

6.5 Discussion

The data shows that the rapid disappearance of phenol in soil is not contributed to by pre-existing soluble enzymes released by microorganisms or plants in the soil in the absence of phenol (non-induced). Use of the microtiter plate method to determine the disappearance of phenol in induced systems showed that the concentration of phenol was reduced slightly in all sets of experiments including blank samples. In the autoclaved soil experiment, where the decrease is not likely to be contributed by microorganism and plants, the phenol decreased by 5%. In the blank sample, the reduction was recorded to be 4% over 6 hours. This may due to the adsorption to the plastic as this was not seen when glass cuvette were used (see below). However, the concentration decreased by 8.5% in the presence of plant and this was significantly higher ($P< 0.05$) than the concentration decrease in microcosm without plant, which gave a reduction of 6.7%. This may be attributed to the ability of the plant to release enzyme for the degradation of phenol or due to interaction between plant and microorganisms that could degrade phenol. This is because the plant alone may be deficient to complete the degradation of contaminant and needs microorganism to complete the process. Plant roots have also been shown to increase metabolic activity of microorganisms by the excretion of root exudates (Rao *et al*, 2010). Reboreda & Caçador, (2008) also demonstrated that *Spartina*

maritima root can increase enzymatic activity of microbes in rhizosphere, which has potential in phytoremediation in salt marshes.

In experiments without plants, the small reduction in phenol concentration may be due to microorganisms acting alone. They can release extracellular phenol oxidase to effect detoxification of phenolic compounds (Sinsabaugh, 2010). Hence, the association of plant and microorganism influences the remediation of organic contaminants due to their extracellular enzyme effect in the biodegradation process (Gianfreda & Rao, 2004).

Using a spectrophotometer with a glass cuvette, there was no decrease in the blank. Phenol concentration was shown to decrease by up to 12% in the experiment with a plant and 7.4% in absence of plant. There are significantly different in the percentage of reduction. Jha *et al*, (2013) reported that when 0.8 g of roots were incubated with 100 mg L^{-1} of phenol solution for 24 hours 28% of the phenol was removed. This percentage was increased by the addition of H_2O_2. Meanwhile, in the samples which were carried out with autoclaved roots, only 10% removal was obtained but no decrease of phenol was observed in the control that had only phenol solution. This may due to the influence of autoclaved process on enzyme activity.
In this study, although the plant seemed to effect on the degradation of phenol, the enzyme activity was slower compared to the rapid disappearance after addition to the soil. The next chapter will turn to the investigation of wheat ability to uptake phenolics compounds and their pathway within the plant.

Chapter 7: Uptake of phenolic compounds by plant tissue

7.1 Introduction

Finally, having recognised in Chapter 3 that the half-life of phenol in the rhizosphere was reduced in the presence of a plant root and that there are different possible ways for the removal of phenol from the soil by plants, I studied to see if a wheat plant has the ability to absorb phenol compounds from soil.

Flocco *et al*. (2002) reported that plants are able to remove phenol directly by adsorption into plant tissues followed by metabolism or storage in some tissues. Phytovolatilization is another pathway, which involves the evaporation of materials through stomata in the leaves. It is also likely that a combination of several processes occurs in some plants (Kang, 2014). This chapter was aimed at examining how wheat plants remove phenol from the soil. In addition to that, syringic acid, a physiological phenolic acid that has been identified as a potential allelochemical in several crops such as wheat and barley (Hura *et al*., 2004), was also studied. The concentration of such compounds in plant tissue is influenced by changes of environmental factors, such as temperature and the nutrient content of soil (Oueslati *et al.*, 2009). Some phenolic compounds are classified as plant secondary metabolites and occur in the soil from root exudation and as the residues of plant decomposition (Zhou *et al*., 2014). Syringic acid is one of the main phenolic compounds detected in wheat bran. Moreover, 17 day-old wheat seedlings, have been reported to exudate some phenolic acids including syringic acid (Oueslati *et al.*, 2009).

Two phenolics were chosen to represent two different "types". Phenol is a classic example of a waste product of human activity (Flocco *et al.*, 2002). Meanwhile, syringic acid is a biological compound which is easily metabolized. In this study, syringic acid and phenol were added separately to soil in the presence and absence of wheat plants to compare their behaviour in the soil (chapter 3 and 5). Subsequently, the concentration of phenol and syringic acid in the roots and leaves were determined. From this, it proved possible to determine some characteristics of the pathways of the phenolics through and out of the plant.

Two different methods were used to sample plant tissue. In the phenol experiments (7.2), bulk root and leaf material was extracted using liquid nitrogen and centrifugation (section 2.12.1). These data, therefore, refer to all the cell types of the relevant organ as well as extracellular material. As shown in chapter 4, a micro method is suitable for syringic acid (but not for

phenol). A glass microcapillary (section 2.12.1) was used to sample individual root and leaf cells in the syringic acid experiments (7.3) (Tomos & Leigh, 1999). These data refer to the content of epidermal and cortical cells of the relevant organ. They will be dominated by vacuole contents.

7.2 Phenol concentration in wheat roots and leaves

7.2.1 Uptake by root

Figure 7.1 illustrates the behaviour of phenol in the root and leaves of wheat plants that had been fed phenol via the rhizosphere. Roots were first extracted (2.12.1) after 30 minutes of addition of phenol to the soil microcosm, at which time the concentration of phenol in the root was 1.3 mM (Figure 7.1A). Subsequently, the concentration of phenol in the root decreased steadily over the experiment time of 24 hours with a $t_{½}$ of approximately 1.8 hours. The trendline in Figure 7.1 is hand drawn and represents the best estimate of the time course of the data. It has been assumed that the behaviour is pseudo exponential.

7.2.2 Uptake by leaves

Leaves from the same plants were extracted (2.12.2). Leaf sample extracted at 30 minutes showed no detectable level of phenol. It takes time for the chemical to travel from the roots to the leaves. At three hours, high levels of phenol were detected in the leaves (Figure 7.1B). This result supports the hypothesis, that wheat plants are capable of taking up phenol from soil through the roots into the leaves. Note that leaf phenol concentration exceed that of the root and rhizosphere (Table 7.1) by 24 hours. As in Figure 7.1A, a hand drawn trendline has been included. This allows estimates and comparison of the rate of changes of concentration at different times.

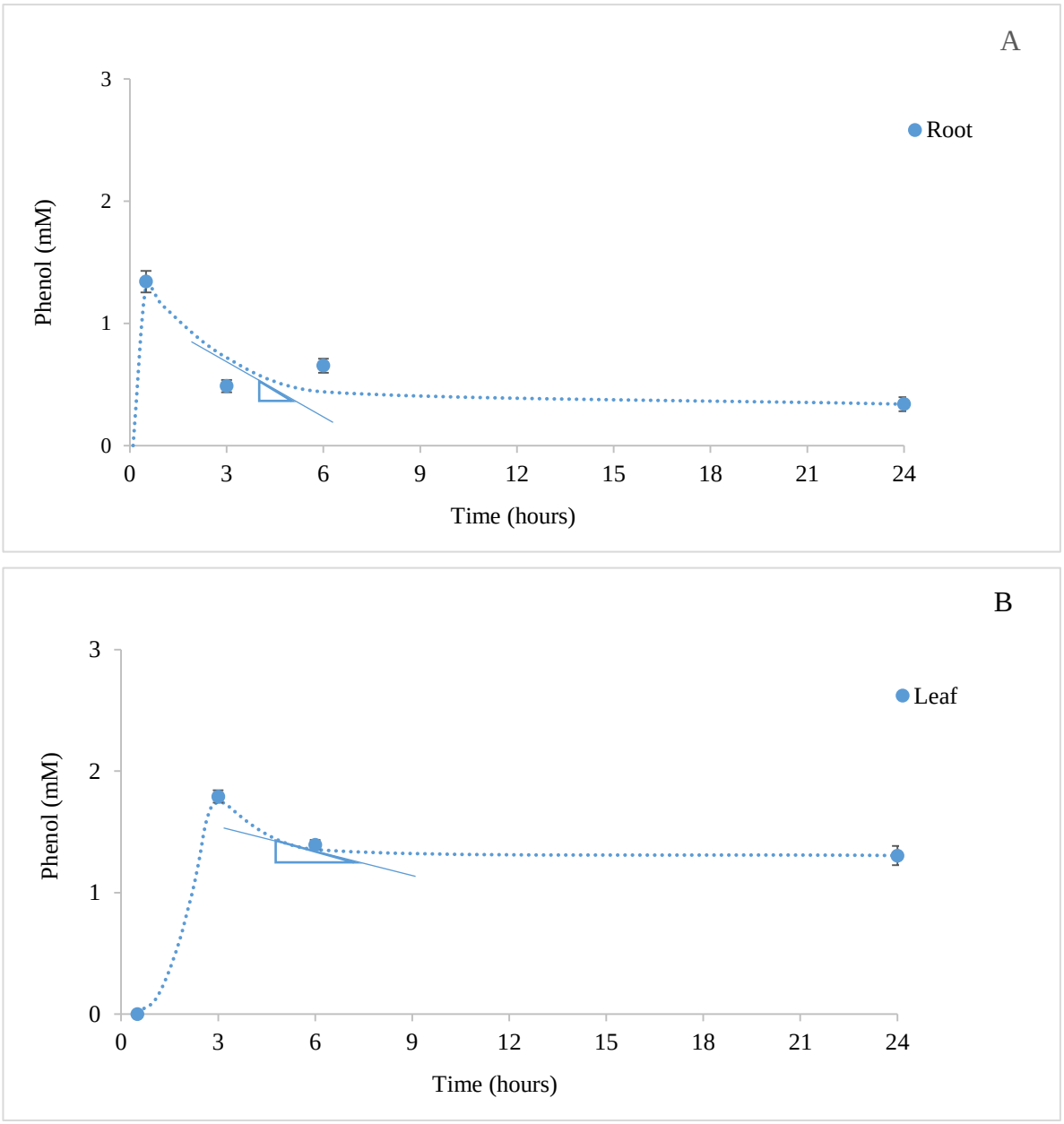


Figure 7.1 Concentration of phenol in wheat root (A) and leaf extracts (B) at varying times after the addition of 20 mM phenol to soil microcosms. The initial phenol concentration in soil moisture was 5 mM (section 3.3.1). Values represent the mean of three replicates, bars represent standard error (SE). [See text for explanation of trend lines]. In (A) a trend line gradient is included to present the rate of change of phenol concentration at 3 hours. This was fitted by eye. The use of this and similar gradients is described in section 7.4.1.3. Note on trend line usage: the small number of points in each case makes the use of trend line speculative. However, the line for leaf (B) describes a not-unexpected trajectory that include all points. In contract, using all the root (A) data points would result in a more complex trajectory. The trend line is a subjective one that offers a relatively simple behavior, comparable with that of the leaf (filing followed by emptying) that is the basis of the discussion the text.

7.3 Syringic acid concentration in wheat roots and leaves

7.3.1 Uptake by root

A glass microcapillary was used (section 2.12.2) to enable a single cell samples of cellular fluid to be obtained from the root. The samples were from epidermal and cortical cells of young, expanded roots. The samples were taken from 30 to 60 minutes after the soil was sprayed with 20 mM syringic acid. Results obtained using this technique reflect the quantity of chemicals (eg: syringic acid) absorbed into the cell, without being affected by contamination from the surface area or soil around the root. No syringic acid was detected in control samples of wheat roots that were not sprayed. Approximately 6 mM syringic acid was detected in the root, but this disappears rapidly with a $t_{½}$ of under 2 hours (Figure 7.2).

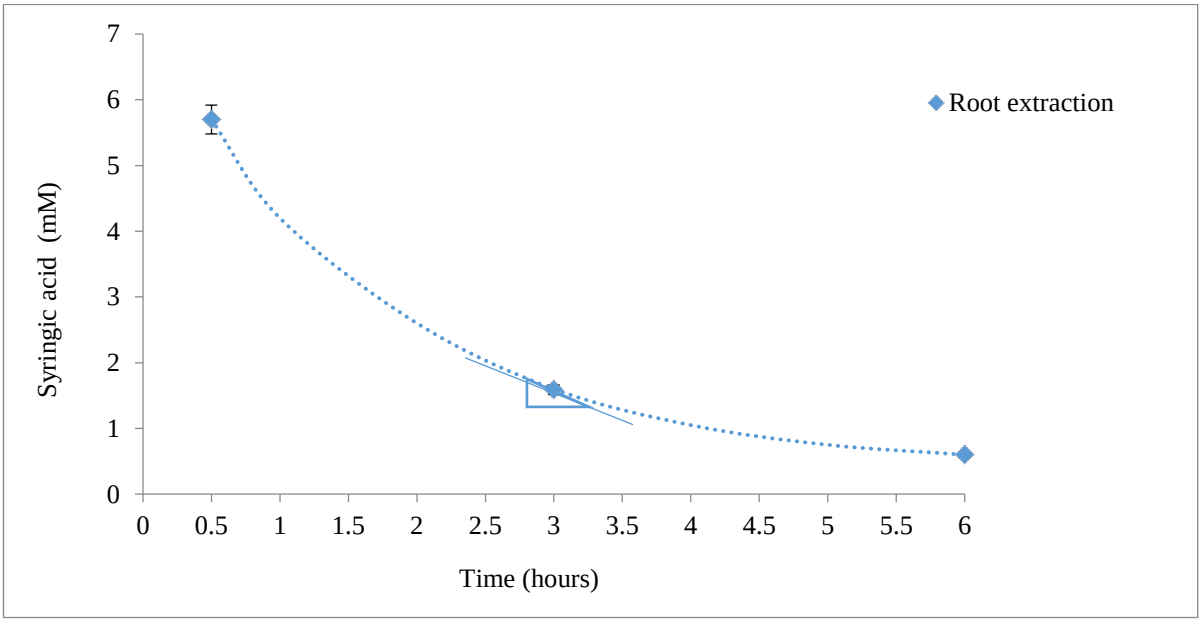


Figure 7.2 Concentration of syringic acid in wheat root cortical cells at varying times after addition of 20 mM to soil microcosm. (Each time point covers sample taken over a 30 min period). Data are mean value ± SE (n=9).

7.3.2 Uptake by leaves

Leaf cells were again sampled using microcapillary (2.12.2) and analysed before spraying (control) and 30 minutes after spraying the microcosm (section 2.7.1). Approximately 1 mM syringic acid was detected in cells of 5 day-old wheat leaves from the microcosm without spraying (control) Figure 7.3. There is no significant difference ($P > 0.1$) in levels of syringic acid content between leaves from microcosms that were not sprayed and microcosms that were sprayed (Figure 7.3).

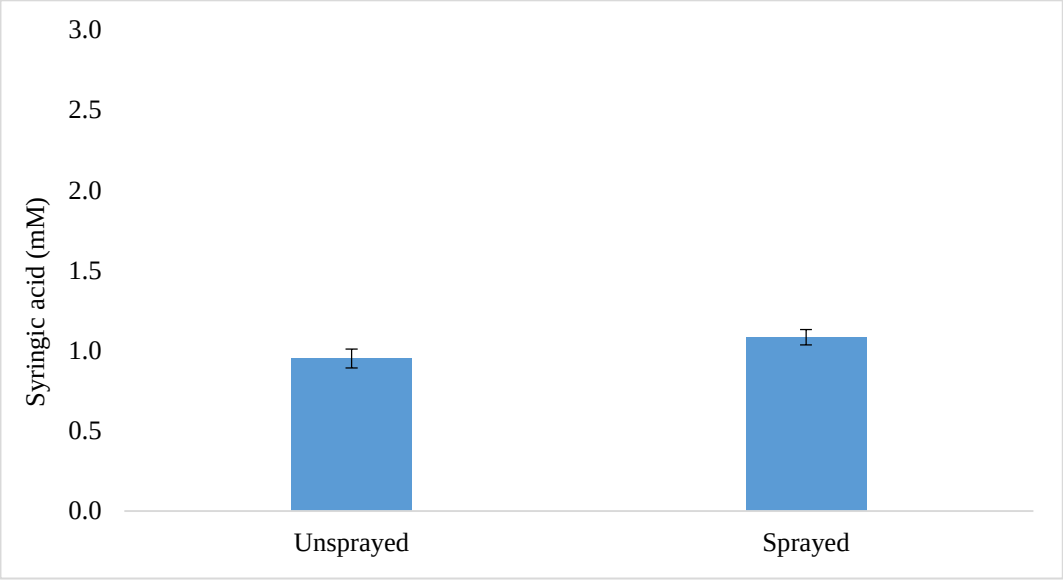


Figure 7.3 Syringic acid content in individual leaf cells of untreated and treated wheat plants. Syringic acid content in leaves 30 minutes after spraying into soil microcosm was compared with control (un-sprayed) plants. There is no significance difference in syringic acid contents between untreated and treated leaves (P >0.1). Results shown are replicates from nine plants. (SE= 0.13 mM and 0.16 mM).

The same experiment was repeated with different plants at different intervals over a 24 hour period, in order to investigate any longer term behaviour of syringic acid in the wheat leaf (Figure 7.4). Although there was a substantial difference in absolute values (Figure 7.4) from the previous experiment (Figure 7.3), there was no significant different in the concentration of syringic acid in leaves of sprayed and unsprayed microcosm. In conclusion, we can say that, in contrast to phenol, there is no evidence to show that syringic acid it is rapidly transported to the shoot.

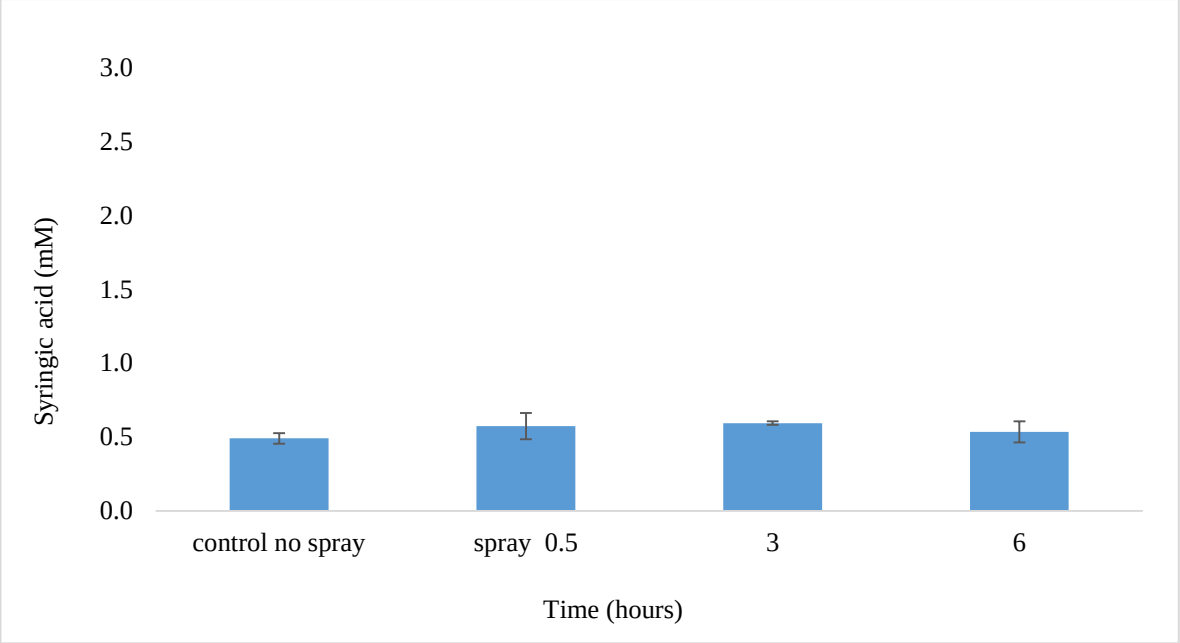


Figure 7.4 Syringic acid concentration in leaf cells after the addition of 20 mM soil microcosm. Analysis was carried out at intervals over 6 hours. There is no significant difference between plants that had the microcosm sprayed with syringic acid and unsprayed control microcosm. (Bars represent sd. n=3).

7.4 Conclusion and discussion

Phenol and syringic acid undergo different processes and follow different physical and metabolic pathways in a plant.

7.4.1 Phenol

It was noted, that in soil with a plant, the phenol concentration declines considerably over time from 5 mM at 0 hour to 0.4 mM at 24 hours (chapter 3). This time course reduced the availability of phenol to the plants as time progressed.

The root is the first portal of entry into the plant. We expected the plant to absorb phenol into the roots and carry it to the leaves by transpiration. The experiments described in this chapter show that phenol is found in both roots and leaves very soon after addition to the rhizosphere but their rates were different. The speed to the leaf (over a distance of several cms) suggest that phenol uptake and translocation are due to transpiration driven bulk flow.

The transpiration rate in wheat was measured to be approximately 0.04 ml / hour (chapter 2, section 2.6.2) from 100 mg of leaf and 40 mg of root. (To facilitate calculation, water is

assumed to have a density of 1g/ml and the transpiration rate is expressed as g/h). At time zero the concentration of phenol in the soil was 5 mM (Figure 3.11, Chapter 3). From equation 7.1, it was estimated that the change of phenol concentration entering the plant (flow rate) expressed as the rate of change of concentration would be approximately 5 mM /hour in the root at time zero (Table 7.1).

Rate of change of concentration =

$$\frac{\text{Transpiration rate (g/h)} \times \text{Phenol concentration in soil (mM) for each point of measurement}}{\text{Root weight (mg)}}$$

Equation (7.1)

Similarly, the equivalent expression for the leaf is:

Rate of change of concentration =

$$\frac{\text{Transpiration rate (g/h)} \times \text{Phenol concentration in root (mM) for each point of measurement}}{\text{Leaf weight (mg)}}$$

Equation (7.2)

Time (hours)	Concentration of phenol (mM)				Calculated rate of concentration increase (mM/h)	
	Soil (without plant)	Soil (with plant)	Root	Leaf	Root	Leaf
0	4.5	5	0	0	5	0
0.5	NA	NA	1.30	0	NA	0.50
3	3.60	3	0.50	1.79	3	0.20
6	3	2	0.70	1.39	2	0.28
24	1.80	0.40	0.30	1.31	0.40	0.12

Table 7.1 Calculated rate of phenol increase in wheat root and leaf. The table shows phenol concentration in soil (from Figure 3.1and Figure 3.11), root and leaf (Figure 7 .1 A&B) of plants measured after spraying phenol in the microcosm. This flow rate was calculated based on transpiration rate (equations 7.1& 7.2).

The concentration of phenol detected in the root was higher in the beginning but gradually decreased, probably due to the transportation to the leaf or it undergoing metabolic process in the root. It takes longer for leaves to have detectable quantity of phenol compared to the root, as leaves are further away compared to root, which is in the soil itself. It takes only 30 minutes for root to accumulate 1.3 mM of phenol and that declined to 0.3 mM at 24 hours (Figure 7.1) with a $t_{½}$ approximately 1.8 hours. Meanwhile, no phenol was detectable at 30 minutes in the leaf. The phenol transported via root to the leaf at 3 hours amounts to 1.79 mM. At the same point in time, analysis in root showed 0.5 mM phenol. Comparatively, phenol from the leaves was not removed to a lower level, unlike in the root. This could be due to the leaf being able to tolerate the toxicity of phenol at these levels. The amount of phenol in the soil depletes over time and the availability of phenol in soil also limits the availability for transpiration through the leaf. Table 7.1 shows the availability of phenol for absorption into the plant root as well as leaf as per reading obtained from the study.

There are three possibilities of what could occur to phenol in leaves. It may be metabolised or stored in the leaf, which would be a positive results for phytoremediation. Alternatively, phenol is a volatile compound and it could evaporate from the leaves. This would contribute to pollution through the air and not be a desirable outcome. Beck *et al*. (1996) reported that edible leaves and fruit could be contaminated by such volatile compounds by volatilisation and transportation through the air. In order to attempt to quantity each of these fates, using the data in this chapter, a simple multi-compartment model was set up (Figure 7.5).

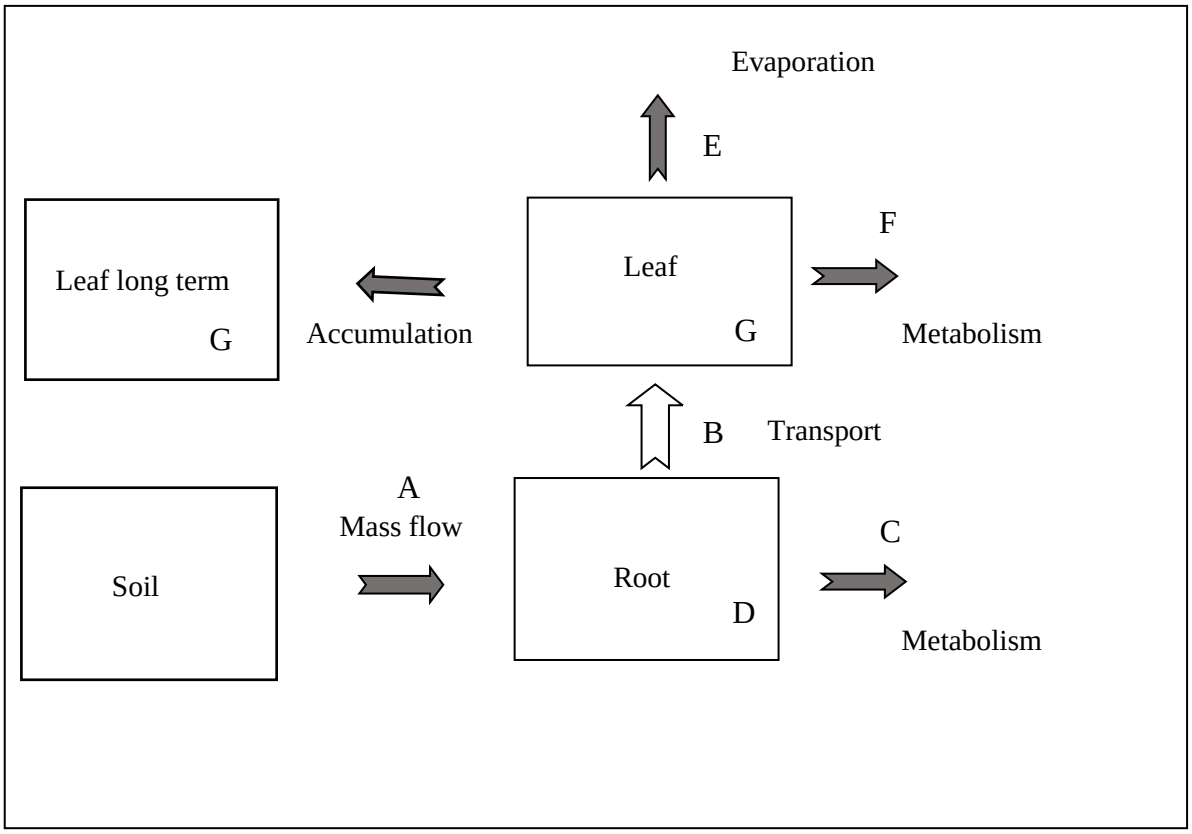


Figure 7. 5 Schematic representation of mass flow of phenol from microcosm into the root and leaf. The letters represent the rate of each step of the process involved. Total amount of phenol flowing into the root (A) is the sum of the amount transported to leaf (B) , the amount metabolised (C) and the rate of the increase in the root (D). {A= D+ (B+C)}. The total transported to leaf (B) is sum of evaporated phenol (E), metabolised (F) and the rate that increase in the leaf (G). {B= G+ (E+F)}.The mass balance is as shown in Table (7.3). (Note than G appear to be the resultant of two compartments-see below).

7.4.1.1 Phenol entering the plant

In order to calculate how much phenol entered the plant, phenol concentration measured in soil (from Figure 3.11 and Table 7.1) and the measured transpiration rate was used (Figure 7.5). The approximation of phenol concentration that was expected from Table 7.2 to enter the plant within each hour was calculated by the following equation (assuming phenol uniformly distributed in the tissue):

Flow rate (µmoles/h) =

[Phenol concentration over each hour interval in soil] × [Transpiration rate]

Equation (7.3)

Time (hours)	Transpiration rate × Phenol in soil (Figure 3.12)	Increase phenol (µmoles/h) during each 1 hour time period
0 -1	0.04×4.50	0.18
1- 2	0.04×4	0.16
2- 3	0.04×3.50	0.14
3 - 4	0.04×3	0.12
4 - 5	0.04×2.50	0.10
5 -6	0.04×2.25	0.09
Total 0 - 6		0.79

Table 7.2 Predicted amount of phenol entering the wheat plant over 6 hours. The increase of phenol concentration during each hour time in the plant based on the transpiration rate and phenol concentration in soil over each hour. At 6 hours the total of expected phenol to enter the plant = 0.79 µmoles.

At 6 hours, the summation of phenol that was expected to enter the plant = 0.79 umoles if equally distributed. The plant weight is approximately = 200 mg → (= 0.2 g) = (0.2 ml). Therefore the concentration would be:

$$\frac{0.79\times10^{-6}\ \text{Moles}}{0.2\ \text{ml}}$$

$= 3.95\times10^{-6}$ Moles/ml.

$= 3.95\times10^{-3}$ Moles/L.

However, from this predicted amount of phenol (3.95 mM) only about the quarter (1 mM – average of 0.7 and 1.39 mM)) was detected in the plant tissue (root and leaf) at 6 hours (Table 7.1). There are three possibilities, phenol may have evaporated, metabolized or phenol did not enter the plant from the soil and the water was traveling faster than phenol.

A consideration of the fate of absorbed phenol and the point at which it is exhausted in the soil is given in the next section.

7.4.1.2 Quantity of phenol detected in plant tissue

The total phenol uptake by the plant at 3, 6 and 24 hours is as shown in Table 7.3, these values were derived and calculated using data in Figure 3.11 A (as estimated in Table 7.2 for 0-6 hours) and the transpiration rate (0.04 ml/h). For example, in Table 7.2 the total amount of

phenol entering the plant at 3 hours is 0.48 µmoles. The amount of phenol in the root at 3 hours can be calculated from phenol concentration in the root (0.5 mM) (Table 7.1) and the weight of root (40 mg; section 7.4.1). Example (at 3 hours) of calculations for phenol in plant root tissue is as shown below:

= 0.5 mM × 40 mg of root

Assuming that 40 mg of root is mainly water, this is equivalent to 40 µl (density of water ~1 g/ml).

= 0.5 mMoles /L× 40 µl

= 20 $\times 10^{-6}$ mMoles

= 20 $\times 10^{-9}$ Moles

= 0.02 µMoles

The amount of phenol in leaf at 3 hours can be calculated from phenol concentration

(1.79 mM) and the weight of leaf (100 mg) as below:

= 1.79 mM × 100 mg of leaf

Again, assuming 100 mg of leaf ~100 µl

= 1.79 mMoles /L× 100 µl

= 179 $\times 10^{-6}$ mMoles

= 179 $\times 10^{-9}$ Moles

= 0.179 µMoles

The amount of phenol thought to have entered the plant but that is not detected in the plant at 3 hours can be calculated from the total amount of phenol entering the plant (0.48 µmoles) minus the amount detected in the root and leaf.

= 0.48 – 0.02 - 0.197

= 0.263 µMoles

This shows 0.263 µMoles (approximately 55%) of phenol disappeared per plant in 3 hours. 0.02 µMoles (4%) was detected in the root, meanwhile 0.197µMoles (41%) phenol was detected in the leaf. The 55% that is lost ends up being evaporated into the atmosphere or

degraded in the plant (Figure 7.5). We cannot distinguish between these. Calculations were also carried out for 6 and 24 hours and the results presented in Table 7.3.

Interestingly, the phenol uptake, dispersion and disappearance appear to vary at different sampled time (Table 7.3).

Time (hours)	Total (µMoles)	Root (µMoles)	Leaf (µMoles)	Evaporated or degraded (µMoles)
3	0.48	0.020 (4%)	0.197 (41%)	0.263 (55%)
6	0.79	0.028 (3.5%)	0.14 (17%)	0.622 (80%)
24	1.87	0.012 (0.64%)	0.131 (7%)	1.727 (92%)

Table 7.3 Quantity of phenol uptake and dispersion in plant tissue.

At 6 hours the phenol uptake increased to 0.79 µMoles and proportionately the quantity of phenol which has disappeared also increased to approximately (80%). The quantity of detectable phenol decreased in both root (3.5%) and leaf (17%).

Similar trend is also seen when sampled at 24 hours. The quantity of phenol uptake into plant further increased to 1.87 µMoles, and the phenol that has disappeared increased approximately to (92%). Meanwhile, the quantity of detectable phenol in root decreased to (0.64%) and in the leaf (7%) respectively.

This shows an increasing net uptake of phenol into plants with time. However, this increase does not proportionately increase the amount of detectable phenol in plant tissue (leaf and root). Interestingly, the more phenol is up taken the more is the quantity that disappears.

The remaining of phenol in soil at 24 hours was only 2% from the initial amount that was added to soil (20 mM) and it seemed to be exhausted after that.

7.4.1.3 Phenol behaviour in root and leaf

Behavior of phenol in leaf was different from the root and within it two compartments could be discriminated as a rapid turnover and a long turnover change of phenol concentration. (Figure 7.6). The rapid compartment emptied within 6 hours. The leaf did not seem to have

increased its concentration of phenol after reaching the maximum of 1.79 mM at 3 hours. Although the level seems to decline in the root. This quantity in leaf was almost 3 times the quantity in root at the same time. This suggests that the phenol concentration increased initially due to transpiration and the amount of phenol available in soil. The leaf phenol then dropped to approximately 1.39 mM with $t_{½}$ approximately 1.3 hours (Figure 7.6). This was similar to root behaviour. This drop might be attributed to evaporation rather than metabolism. The second compartment appeared to be a long term store where the concentration of phenol remains at 1.3 mM throughout (Figure 7.6). It appears that the leaves were able to remove the phenol levels down to 1.3 mM and not below that. Phenol was not removed from plant tissue after 24 hours. This could be because this concentration is tolerable to the plant or this level does not cause significant damage to the plant. However, high levels of phenol accumulated in cells can cause serious damage and death to plants (Flocco *et al*., 2002). I attempted to interpret these result by comparing the best estimate of phenol flux at different points in the pathway from rhizosphere to leaf (Figure 7.5) as they change with time.

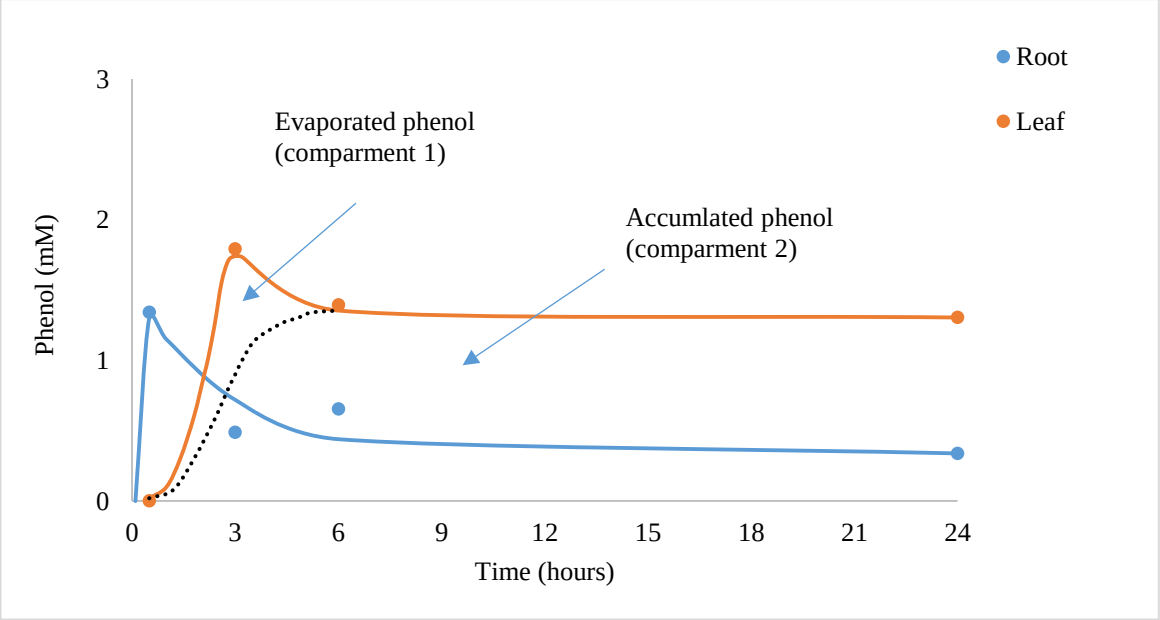


Figure 7.6 Compartments of phenol concentration detected in wheat root and leaf. Data in Table 7.1 presented in a graph format. The peak of phenol concentration detected in root and leaf is shown. The phenol is first absorbed into the root very quickly and reaches the highest measured level in 30 minutes, it then decreases dramatically probably due to transportation to leaf as well as metabolized by root. During this period, the phenol is transported to leaf. At 3 hours the concentration of phenol increases dramatically (compartment 1) but then quickly drops to steady concentration (of 1.3 mM). The concentration in leaf is almost 3 times the concentration in root and seemed to be long term store (compartment 2- below the dotted line).

7.4.1.4 Mass balance

In chapter, 3 we have discussed that the presence of wheat plant increased the removal of phenol compared to the (microbial activity of) control soil alone. Here we have calculated the mass balance of phenol that is taken up and metabolised by the wheat plant. Table 7.4 and Figure 7.5 show mass balance of phenol in the plant. The phenol that enters the root to the leaf is represented in the diagram (Figure 7.5). The phenol from the surrounding enters the plant via the root. The flow rate (A) is represented by the gradients of phenol concentration at each point of time measurement in soil (Figure 3.11). Meanwhile, an estimate of the maximum value of A was made assuming that phenol and water entered the root in the same proposition as found in the soil. For this the equation used was:

A = [Soil concentration of phenol] x [Transpiration rate] *Equation* (7.4)

e.g. a 6 hours phenol concentration = 2 mM (Table 7.1).

A = 2 mM x 0.04 g/h

= 2 $x10^{-3}$ Moles/kg x 0.04 g/h

= 2 $x10^{-6}$ Moles x 0.04/h

= 0.08 $x10^{-6}$ Moles/h. (Table 7.4 at 6 hours).

Some (if not all) the phenol that is drawn from soil (A) into the root would be further transported to leaf (B). However, some quantity of phenol would result in changing root concentration (D) and some would be metabolized in the root (C). The mass of phenol transported to leaf also faces a similar fate.

Presumably, some of the phenol escapes the leaf by evaporation process (E) and some undergoes metabolism in the leaf (F). Some amount that remains in the leaf (G).

The gradient of the root and leaf phenol concentration time course (Figure 7.1) was used to get the values for the rates D and G in Table 7. 4 (A&B). This gradient value were multiplied by the weight of the root and leaf to generate the rates.

Root increase rate = Gradient × Weight of roots (mg). *Equation* (7.6)

e.g. at 6 hours in the root (Figure 7.1A and Table 7. 4):

The gradient = 0.0056 mMoles/L.h.

= 0.0056 x 10^{-3} Moles/L.h.

40 mg = (0.04 g) = 0.04 ml

D = 0.0056 x 10^{-3} Moles/L.h x 40 L x10^{-6}

= 0.224 x10^{-6} x 10^{-3}

= 0.00022 x10^{-6} Moles/h.

It in the root, therefore follow that (B+C) = A –D.

The same formula was used to calculate the situation for leaves, where the weight of root is replaced by weight of leaf (Table 7.4 B).

(E+F) = B – G.

There were two estimations for B. First one was made assuming that there was not metabolism in the root (C = 0) and this was used to calculate B (Table 7. 4). The second separate estimate of B not having to assume C to be zero. The equation for this:

B = concentration in root x transpiration rate. *Equation* (7.5)

A) Mass flow in root

Time (hour)	A (moles/h)	D (moles/h)	B+C (moles/h)	Gradient (moles/L.h)
0	0.2×10^{-6}	0.104×10^{-6}	0.1×10^{-6}	2.6×10^{-3}
0.5	0.18×10^{-6}	0.014×10^{-6}	0.16×10^{-6}	0.35×10^{-3}
3	0.12×10^{-6}	0.0012×10^{-6}	0.1188×10^{-6}	0.03×10^{-3}
6	0.08×10^{-6}	0.00022×10^{-6}	0.079×10^{-6}	0.0056×10^{-3}
24	0.016×10^{-6}	0.0016×10^{-6}	0.0144×10^{-6}	0.042×10^{-3}

B) Mass flow in leaf

Time (hour)	B (moles/h)	G (moles/h)	E+F (moles/h)	Gradient (moles/L.h)
3	0.1188×10^{-6}	0.018×10^{-6}	0.1×10^{-6}	0.18
6	0.079×10^{-6}	0.0059×10^{-6}	0.037×10^{-6}	0.059

Table 7. 4 The mass of phenol entering the root and leaf quantified from Table 7.1 using the concentration of phenol in soil, root and leaf. As shown in Figure 7.5 the letters represent the mass flow of phenol through the plant in each step of the process. Total amount of phenol flowing into the root (A) is sum of the amount transported to leaf (B) , the amount metabolised (C) and the rate of increase in the root (D). {A= D+ (B+C)}. The total transported to leaf (B) is sum of evaporated phenol (E), metabolised (F) and the rate that increase in the leaf (G). {B= G+ (E+F)}.

The findings lead to the conclusion that phenol from the soil goes through the plant via root, then to leaves followed by evaporation through apoplast into the air. Phenol is assumed to be taken into the plant by transpiration. Others have reported that the uptake of phenol by mature soybean plants was due to transpiration (McFarlane *et al*., 1987). Part of the phenol is likely to be metabolised (this might to be in cytoplasm) and part of stored in vacuoles of the leaf cell. It most likely that most of the phenol was removed by evaporation. Ucisik &Trapp (2006) reported similar suggestion. In their studies phenol was detected in the root and leaves of Willow trees (*Salix viminalis*) after addition to the sand and hydroponic solution. They suggested the phenol uptake process could occur via advection with the transpiration water. Willow trees caused a fivefold loss of phenol in the rooting media. However, only 4.53% was recovered from the plant, suggesting loss of phenol by metabolism or volatilization from leaves. When low levels of phenol, (10 to 100 mg/L; approximately 0.1 to 1 mM) were added to the solution no parts of the plant had high levels of phenol. The highest concentration of phenol was detected in root and stem when higher concentration of phenol solution is added in solution (250 mg/L to 500 mg/L; which equal approximately 2.5 to 5 mM). However, when the concentration exceeded 500 mg/L the plants did not survive, meaning the phenol concentration exceeded the tolerable level of the plant. In this study, 20 mM was added to wheat microcosm. Due to the dilution and adsorption(discussed in chapter 3), the initial soil moisture concentration at zero time was 5 mM. It was rapidly removed from the soil in the presence of wheat and the leaf seemed be able to tolerate 1.3 mM of phenol.

Phenol decontamination by mung bean (*Phaseolus aureus*) and wheat (*Triticum vulgare*) seedling has been studied by Ugrekhelidze *et al*. (1999). They studied transformation of ^{14}C - phenol during penetration into the plant's root. They suggest that phenol conjugates with low-molecular-weight peptides. The research assumed that the hydroxyl group of phenol and the functional groups of peptides play a role to achieve the conjugation. It is suggested that the main pathway for detoxification of phenol is likely to be caused by conjugation with low-molecular-weight peptides. This might correspond with the suggestion that some amount of phenol in wheat was metabolized in root (C) and leaf (F) in (Figure 7.5) after the addition to soil.

Flocco *et al*. (2002) also reported that plants help removal of phenol from solutions by directly absorbing the phenol into the tissues where they metabolize or store them. Removal of phenol by a plant could occur by absorption into plant tissue by mechanism of conjugation with endogenous compounds and by storage in vacuoles. Residues can also bind covalently to lignin

and cell wall components and by oxidative degradation catalysed by several types of oxidative enzymes localized in the cytoplasm and organelles.

The metabolism of phenol in plants is related to oxidoreductive enzymes and they can be used as non-specific biomarkers of environmental pollution. Their increased levels are believed to be a reaction by plants to free radical oxidation in order to protect plant cells. Detoxification of phenol is assisted by plant peroxidises by coupling of phenol with other parent molecules or plant structures like lignin (Flocco *et al.,* 2002).

7.4.2 Syringic acid

Syringic acid behaves differently to phenol. Syringic acid concentration detected in roots almost immediately after addition to the soil was higher compared to phenol. However, it declined rapidly from 5.7 mM at the start to 1.59 mM at hour 3 and to 0.6 mM at hour 6. The same was not found in the leaves. The leaves seems to have a constant level syrigic acid, which is about 0.6 at hour 0 and 0.5 mM over 6 hours. Uptake due to transpiration of syringic acid was calculated at 7 mM in root (Table 7. 5) if syringic acid followed the same behavior as phenol. Potential flow rate was calculate using these equations:

Rate of change of concentration =

$$\frac{\text{Transpiration rate (g/h)} \times \text{syringic acid concentration in soil (mM)}}{\text{Root weight (mg)}} \qquad \textit{Equation } (7.7)$$

Similarly, the equivalent expression for the leaf is:

Rate of change of concentration =

$$\frac{\text{Transpiration rate (g/h)} \times \text{syringic acid concentration in root (mM)}}{\text{Leaf weight (mg)}} \qquad \textit{Equation } (7.8)$$

Time (hours)	Concentration of syringic acid (mM)				Calculated rate of concentration increase (mM/h)	
	Soil (without plant)	Soil (with plant)	Root	Leaf	Root	Leaf
0	8	7	0	0.50	7	NA
0.5	3.50	4.20	5.70	0.60	4	2.28
3	1.40	1.60	1.59	0.60	1.60	0.63
6	0.60	0.70	0.60	0.50	0.70	0.24

Table 7.5 Calculated rate of syringic acid increase in plant tissue. The table shows syringic acid concentration in soil from (Figure 5.1), root and leaf of plants (Figures 7.2; 7.4), which was measured after spraying syringic acid onto the microcosm. This flow rate was calculated based on transpiration rate (equations 7.7&7.8).

7.4.2.1 Syringic acid entering plant

How much syringic acid entered the plant was calculated from the syringic acid concentration in soil (from Figure 5.1 and Table 7.6) for each hour and transpiration rate (Table 7.5; *Equation* 7.9).

Flow rate (µmole/h) =

[Syringic acid concentration change over each hour in soil] × [Transpiration rate]

Equation (7.9)

Time (hour)	Transpiration rate × syringic acid in soil (Figure 5.1)	Increase syringic acid (µmoles/h) during each 1 hour time period
0 - 1	0.04×6	0.24
1 - 2	0.04×4.25	0.17
2 - 3	0.04×3	0.12
3 - 4	0.04×2	0.08
4 - 5	0.04×1.25	0.05
5 - 6	0.04×0.80	0.03
Total 0 - 6		0.69

Table 7.6 Predicted amount of syringic acid entering the wheat plant over 6 hours. The increase of syringic acid concentration during each hour time in the plant based on the transpiration rate and syringic acid concentration in soil over each hour. At 6 hours the total of syringic acid that was expected to enter the plant = 0.69 µmoles

The approximate amount that was expected to enter the plant at 6 hours was 0.692 µmoles if equally distributed in the plant tissue and the plant is equal approximately = 200 mg → (= 0.2 g) = (0.2 ml). Therefore, the concentration would be:

$$\frac{0.692 \times 10^{-6}\ \text{Moles}}{0.2\text{ml}}$$

$= 3.46\times10^{-6}$ Moles/ml

$= 3.46\times10^{-3}$ Moles/L

However, the measured value in leaf and root at 6 hours (Table 7.5) was found to be lower than this expected value. Syringic acid may have been metabolized in the root system and not transported to the leaf or its metabolism balanced the absorption rate. There was no evidence for the uptake of syringic acid into the leaf.

7.4.2.2 Syringic behavior in root

The behaviour of syringic acid inside the plant was different to that of phenol. Initially, the root takes up syringic acid rapidly within 30 minutes, which is similar to phenol and the $t_{½}$ of disappearance was also similar for both of them. It was approximately 1.6 hours for syringic acid and approximately 1.8 hours for phenol inside the root. The rapid disappearance of syringic acid is attributed to metabolism because the data does not indicate uptake by the leaf (Figure 7.7). Meanwhile, intrinsic syringic acid content in leaves found in our study was between 0.5 to 1 mM. The reported bioactivity range is between 0.1 to 1 mmol/ L (Piotrowski *et al.*, 2008).

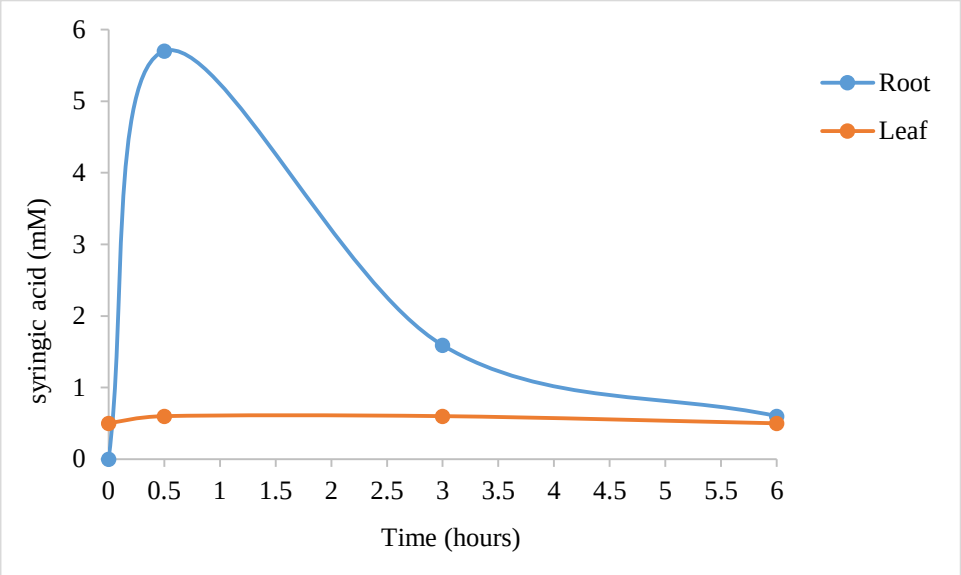


Figure 7.7 Compartments of syringic acid concentration detected in wheat leaf and root. Data in Table 7.5 presented in a graph format. The peak of syringic acid concentration detected in root is clearly shown in the above graph. The syringic acid is first absorbed into the root and reaches the highest level in 30 minutes. Then dramatically decreases probably due to metabolism. On other hand, no increased levels of syringic acid observed in leaf. There is no change in concentration of syringic acid in leaf before and after addition of syringic acid into the microcosm.

7.4.2.3 Mass balance

The mass balance of syringic acid that was taken up and metabolised by the wheat plant (Figure 7.8) was calculated (Table 7.7). The syringic acid mass that enters the root to the leaf is represented in diagram 7.7. The syringic acid from the surroundings enters the plant via the root a flow rate (A) which represents the gradients of syringic acid concentration at each point of the time measurement in soil (Figure 7.2).

Meanwhile A can be estimated as:

[Soil concentration of syringic acid] x [Transpiration rate] *Equation* (7.10)

e.g. a 3 hours syringic acid concentration = 1.6 mM (Table 7.5).

A = 1.6 mM x 0.04 g/h

= 1.6 $x10^{-3}$ Moles/kg x 0.04 g/h

= 1.6 $x10^{-6}$ Moles x 0.04/h

$= 0.064 \times 10^{-6}$ Moles/h.

The data (Table 7. 5) shows no evidence for the transport of syringic acid into the leaf after the addition to the soil. Therefore, the net rate of syringic acid transported into the leaf (B) = 0. Some quantity of syringic acid would lead to the change in the concentration (D) and some would be the rate of syringic acid metabolized in the root (C).

Again, as in the case of phenol, the gradient of the root concentration time course (Figure 7.2) was used to get the value for the rate D in Table 7.7. This gradient values were then multiplied by the weight of the root to generate the rates.

Root Increase rate = Gradient × Weight of roots (mg). *Equation* (7.11)

e.g. at 3 hours in the root (Figure 7.2 and Table 7.7):

Gradient = 0.85 mMoles/L.h.

$= 0.85 \times 10^{-3}$ Moles/L.h.

40 mg = (0.04 g) = 0.04 ml (assuming 1 ml of solution approximately = 1g).

$D = 0.85 \times 10^{-3}$ Moles/L.h x 40 L $\times 10^{-6}$

$= 34 \times 10^{-6} \times 10^{-3}$

$= 0.034 \times 10^{-6}$ Moles/h. (Table 7.7).

In root, (B+C) = (A) – (D).

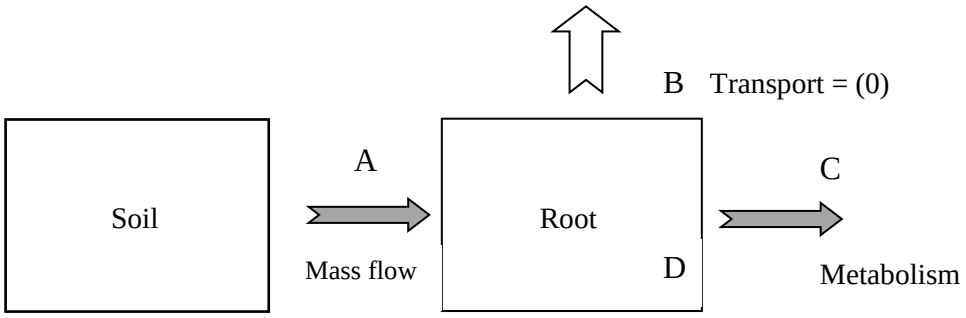


Figure 7.8 Schematic representation of flow of syringic acid mass from microcosm into the root. The letters represent the rates of change of mass in each step of process involved. Total rate of syringic acid flowing into the root (A) is sum of the amount transported to leaf (B) that is assumed to be (0), the amount metabolized (C) and the rate of increase in the root (D). {A= D+ (B+C)}.

Time (hour)	A (moles/h)	D (moles/h)	B+C (moles/h)	Gradient (moles/L.h)
0	0.28×10^{-6}	0.2×10^{-6}	0.08×10^{-6}	6.8×10^{-3}
0.5	0.16×10^{-6}	0.11×10^{-6}	0.05×10^{-6}	2.75×10^{-3}
3	0.064×10^{-6}	0.034×10^{-6}	0.03×10^{-6}	0.85×10^{-3}
6	0.028×10^{-6}	0.016×10^{-6}	0.012×10^{-6}	0.4×10^{-3}

Table 7.7 The mass of syringic acid entering the root quantified from Table 7.4 using the concentration of syringic acid in soil, root. As shown in Figure 7.8 the letters represent the mass flow of syringic acid through the plant in each step of the process. Total amount of syringic acid flowing into the root (A) is sum of the amount transported to leaf (B) = (0), the amount metabolized (C) and the amount of increase in the root (D).

4.4.3 Physical pathway

Both phenol and syringic acid are water soluble, thus are carried easily to the plant system along with water. There are three possible routes water can follow when entering the root system (Raven *et al.*, 1992). Figure 7.9 illustrates the three possible pathway by which water is carried into the plant. The water passes through the soil particle into the extra-cellular parts of the root, which includes the cell wall. As the cell walls are absorbent, the water will flow or diffuse through them. This is called the apoplast pathway. This is the fastest pathway where most dissolved chemicals and water travel along. Then the water could also go through the symplast pathway, which is passing of water through the living cytoplasm of cells via plasmodesmata. Neighbouring cells are connected with each other's cytoplasm by plasmodesmata. Vacuolar pathway is when the water travels from vacuole to vacuole of one cell to the next by osmosis.

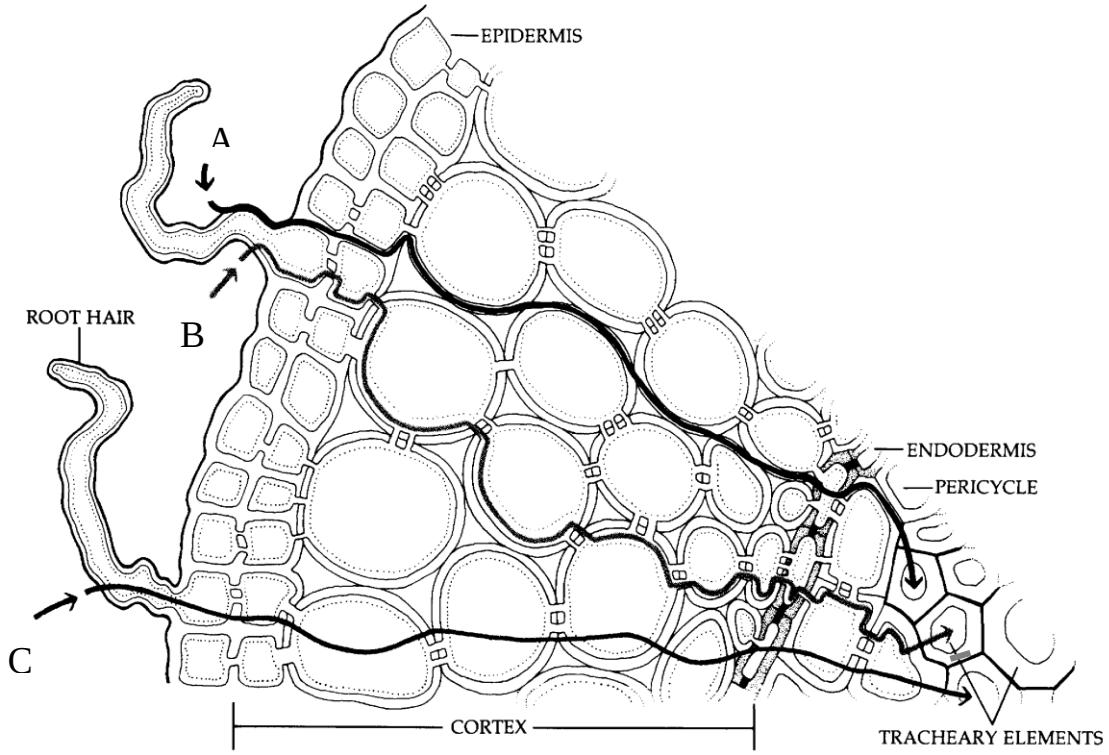


Figure 7.9 Pathways by which water and chemicals are taken through cells in roots of plants. From (Raven *et al.*, 1992). A) Apoplast pathway. B) Symplast pathway. C) Vacuole pathway.

Xylem vessels carry water and water dissolved chemicals within the plant. Phenol, being soluble in water, is also assumed to be able to be carried in the apoplast pathway into the xylem of the plant and to the rest of the plant system including the leaves. According to Steudle & Petersen (1998), solutes in apoplast pathway move by convection with water while in symplast pathway they move by the active or passive permeation through the plasma membrane (and tonoplast in the case of the vacuole pathway). The reflection coefficient of the apoplast is very low which denotes the high permeability of this pathway and phenol is expected to go through the apoplast. In phenol experiments the root was sampled using the macro approach and this would include all pathways. But phenol is assumed to go through the apoplast pathway. If it were assumed that the apoplast pathway presented 20% of the root, according to the result (Table 7.1) we expected within one hour the root would have about 5 mM from the soil if there were no selectivity. But only 1.3 mM was detected. This indictes some selectivity.

Phenol is less polar than syringic acid (see chapter 9) and some is expected to move with water flow along the cell walls due to the transpiration stream in the xylem from the apoplast pathway until it reaches the Casparian strip in the endodermis layer. According to Trapp & McFarlane (1995) all the compounds cannot pass through Casparian strip and are forced to enter the symplast pathway by crossing the plasma membrane and enter the protoplast of the endodermis cell in order to reach xylem for translocation. Phenol may be moved passively through the plasma membrane. Passive movement of chemical into and through a membrane is determined by a property called lipophilicity of the chemical. This is related to partition coefficient (represented by the octanol/water, K_{ow} value). In order for passive uptake to occur, K_{ow} value of 0.5 to 3.0 is required (Flocco *et al*., 2002). Smaller log K_{ow} values implies that a compound is too hydrophilic to go through cell membrane. Phenol has log K_{ow} value of 1.46 (see chapter 4 for disscution), which suggest that the roots can partially adsorb phenol and move across cell membranes by dissolving in phospholipid layer (Flocco *et al*., 2002). However, Steudle & Petersen (1998) report that the main chemical component of Casparain strip in different species is lignin and it is still hydrophilic. This might correspond with the suggestion of water passage through Casparan strip. Similarly, phenol may pass the Casparian strip with the water. In contrast, as syringic acid is more polar, go in the vacuolar pathway, and it may be forced to pass the cells membrane to reach vascular system (Figure 7.9). There is no evidence from our result for the transport of syringic acid to the leaf.

Relatively little is known about the membrane transport of this molecule. Monolignols such as p-coumaryl alcohol are synthesized by peroxidases and laccases in the cytosol and transported to cell wall to produce lignin (Alejandro *et al., 2012*; Sibout & Höfte, 2012). Although the transport process across the plasma membrane is still unclear, they suggested that AtABCG29 and ATP-binding cassette transporters are acting as p-coumaryl transporters for monolignol through the plasma membrane to cell wall, vacuole or Golgi apparatus. It might be expected that similar transport process are involved in the root uptake of these compounds.

Phenolic acids are a recognized defence system in the root. Zengqi Li *et al. (*2012) reported that phenolic compounds were mainly distributed in the vacuoles of the cortex parenchyma cells of roots of *Echinacea purpurea*. In addition, phenolic compounds may go through the cell wall to be stored in the intercellular spaces outside of the cell in order to inhibit the pathogens invading from the apoplast pathway, thus acting as the first defence barrier when the plant is attacked or damaged.

Chapter 8: Determination of industrial soil pollution by phenolic compounds

8.1 Introduction

Phenolic compounds are present in industrial wastes as a result of manufacturing processes such as oil refineries (Basha *et al*., 2010). Benzene is a product from the petrochemical and petroleum refining industries and is one of the components of petrol. These products contain a number of contaminants that are classified to be carcinogenic to humans (Zhang *et al*., 2002). Consequently, humans are exposed to benzene as part of occupational exposure in factories, refineries and other related industries. It is also generally found in petrol, vehicle exhaust and diesel fuel (Celik *et al*., 2003). Phenol is the main metabolite of benzene (Zhang *et al*., 2002) and is an important pollutant due to its toxic nature. This experiment was carried out in order to evaluate the distribution phenol in soil samples collected in Saudi Arabia.

8.2 Phenolic compounds concentration in tested soil

Five samples were collected from different areas of Al-Jouf, in Saudi Arabia. The samples were collected from 2 to 3 cm depth of soil from: industrial area (A), two different petrol stations (B, C), Agriculture soil (D) and a sample from a local park (E) to be used as a control.

A sample of soil (10 g) from each area was mixed with 1.5 ml deionized water separately. Subsequently, the mixture was extracted to obtain soil solution by centrifugation (section 2.8.1). The sample of soil solution was analysed by CZE using 3CP as internal standard.

Only one peak was observed in the soil samples from the industrial site (Figure 8.1). In contrast, two different peaks were detected from the petrol station 1 and 2 (Figure 8.2 & 8.3). These peaks were assumed to be phenolic compounds in the tested samples due to their UV absorbance (at 240 nm). The second peak in (Figure 8.3) seemed to be phenol when compared to the peak of phenol in control sample. Control samples were 20 mM of both phenol and 3CP (Figure 8.4).

In the agriculture soil samples also one peak was observed (Figure 8.5), which might be phenolic compound that had been released in soil by plant or microorganism but does not correspond with syringic acid peak in control sample (Figure 8.6). Meanwhile, no significant UV absorbing peak was observed in the samples from the local park.

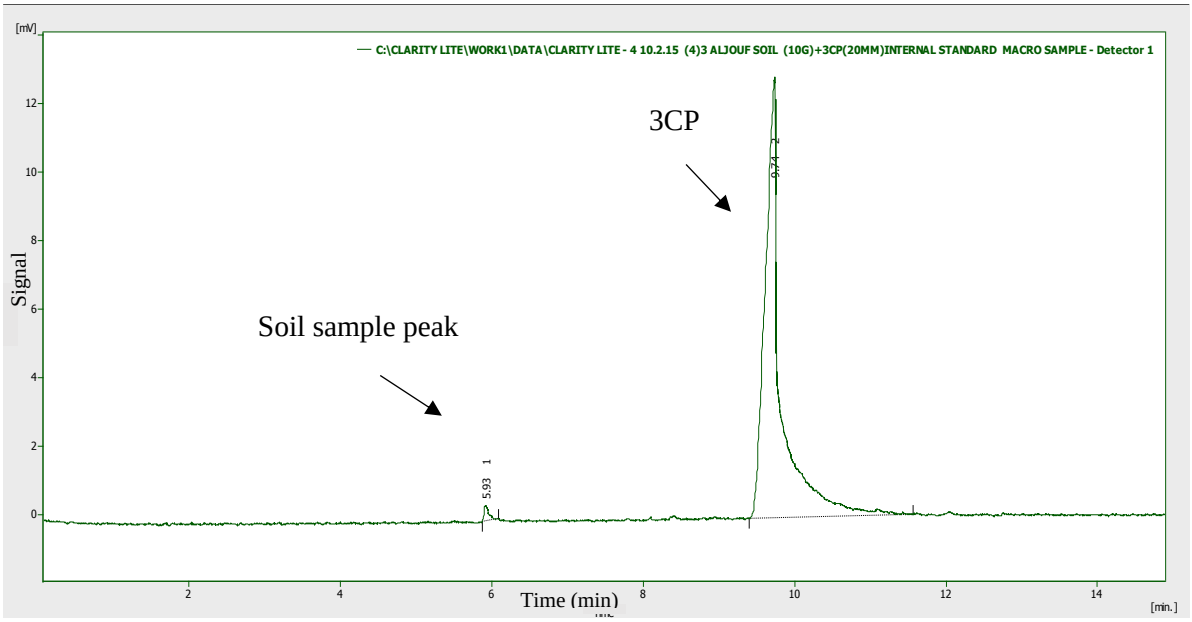


Figure 8.1 Phenolics separated in tetraborate buffer in soil sample from Industrial area using CZE at 240 nm. 3CP was used as internal standard (see text for details).

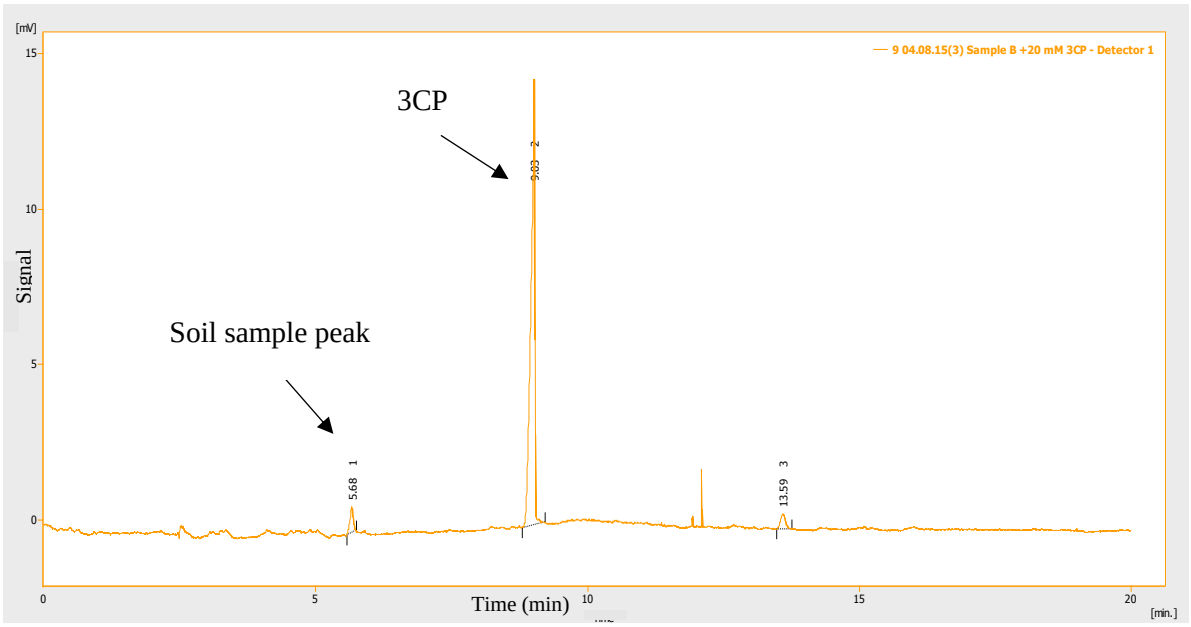


Figure 8.2 Phenolics separated in tetraborate buffer in soil sample from petrol station 1 using CZE at 240 nm. 3CP was used as internal standard.

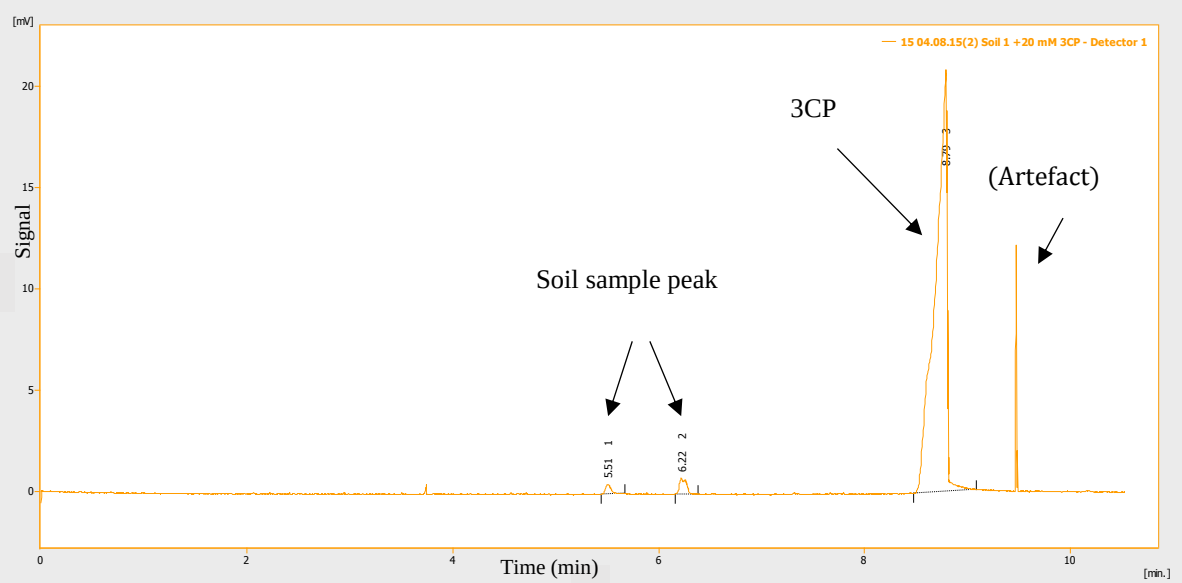


Figure 8.3 Phenolics separated in tetraborate buffer in soil sample from petrol station 2 using CZE at 240 nm. 3CP was used as internal standard.

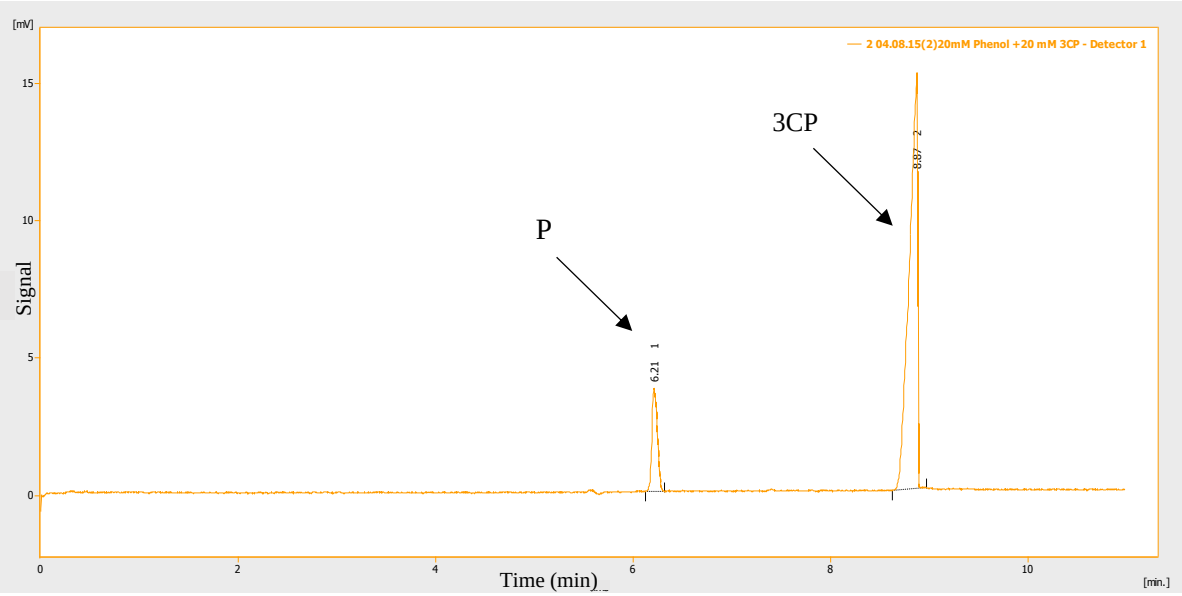


Figure 8.4 Control sample of phenol and 3Chlorophenol separated in tetraborate buffer using CZE at 240 nm.

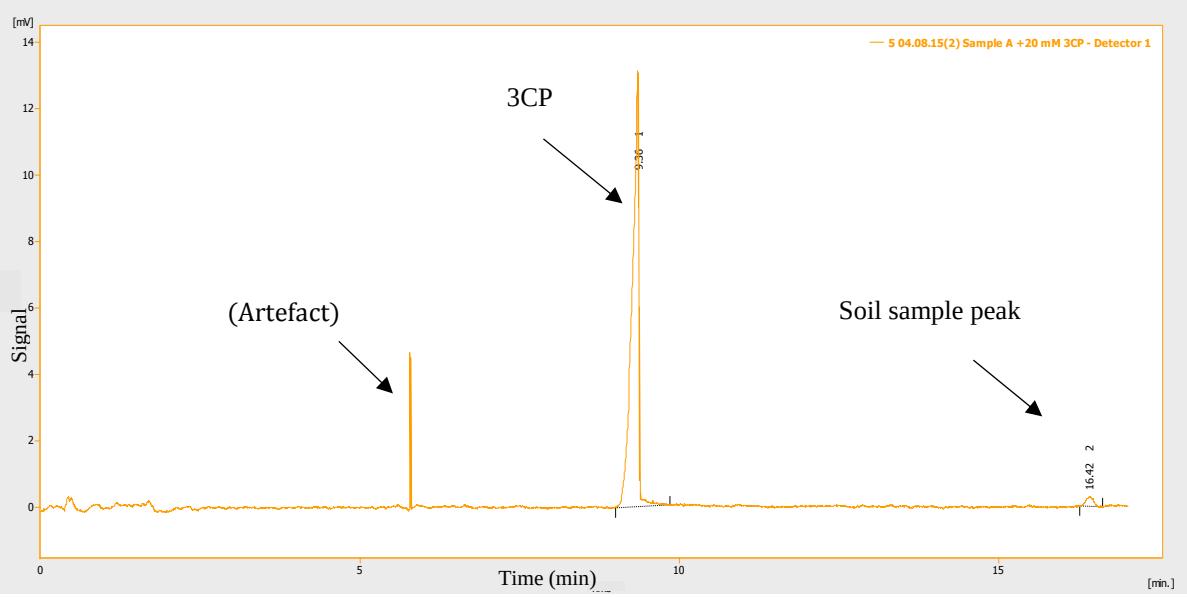


Figure 8.5 Phenolics separated in tetraborate buffer in soil sample from agriculture soil using CZE at 240 nm. 3CP was used as internal standard.

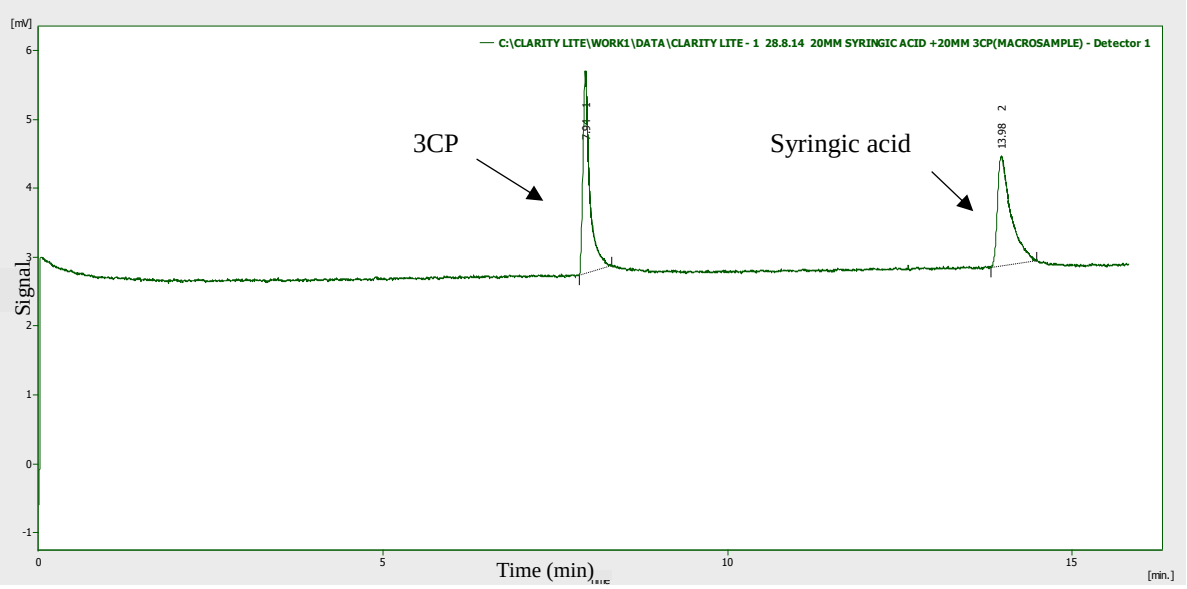


Figure 8.6 Control sample of 3Chlorophenol and syringic acid separated in tetraborate buffer using CZE at 240 nm.

8.3 Discussion

The concentration of monitored compounds were different between the soil samples taken from petrol station and other areas (Figure 8.7). This may refer to their different location and related industrial activity. Sample B petrol station has higher reading compared to the other petrol station due to its location and size. This petrol station is larger as it caters for a bigger population. It seems, the higher concentration of phenolics found in the soil could be directly related to more petroleum products being handled. Baciocchi *et al*. (2003) reported a large number of sites that had been found to be contaminated with hazardous compounds such as phenols. If the initial concentration of these pollutants is high, then it will be toxic for the microbes. Therefore, remediation through the biological processes in such sites may not be achieved.

According to the data in chapter 3, the initial concentration after the addition of phenol (20 mM) to (50 g) soil was 5 mM. This disappeared in 9 hours in the presence of plant in soil and 13 hours in soil without any plant. In tested soil, the accumulation of phenolic compounds in soil was between approximately 2 to 0.5 mM. The high concentration of phenol has an influence on the sufficience of phenol degradation by bioremediation process (El-Khateeb, 2014). Phenol may act as an inhabitor for its degradation when found in high concentration (Saez & Rittmann 1991; Caputo *et al*., 2013). Therefore, the accumulation of these compounds in the tested sandy soil may due to the continuous addition of phenol to the soil with less amount of microbes.

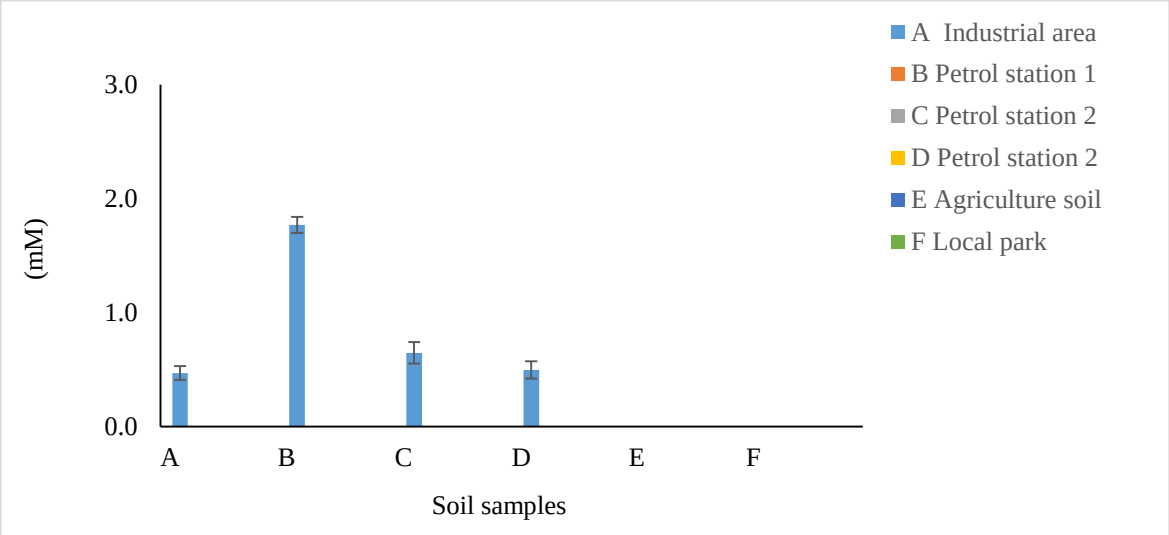


Figure 8.7 Phenolic compound concentration in soil samples collected from different sites: A) Industrial area, B) petrol station1, C) petrol station 2, D) agriculture soil and E) local park. The levels of phenolic compound concentration vary depending on the site from where the soil sample was collected.

Chapter 9: Final Discussion

9.1 Phytoremediation and its current use

Environmental pollution has become an inevitable consequence of human development and material progress. A safe, cost effective and eco-friendly method of restoration by removal and neutralization of these pollutants is vital for the survival of humans as well as other living things on the planet. The technology of using plants for the removal of pollutants, called phytoremediation, seems to be environmentally friendly, and has become important in recent times as an alternate technology in removal of pollutants from soil as well as water (Eapen *et al*., 2005). Many plants have been studied and found to have natural ability to remove pollutants and restore the environment (McCutcheon & Schnoor, 2004).

Phytoremediation process can be efficiently enhanced by plant-associated beneficial microbes (Khan, 2005). This has been demonstrated in phytoremediation researches (Glick, 2003; Chaudhry *et al*., 2005; Gerhardt *et al*.,2009), where heavy metal uptake mediated by plant associated microbes acts directly by altering metal accumulation in the plant tissue or indirectly by shoot and root biomass production. Biogeochemical processes such as translocation, transformation, immobilization, solublization, precipitation, and volatilization further facilitate or enhance phytoremediation process (Rajkumar *et al*., 2012). Phenols and polyaromatic hydrocarbons (PAH) are examples of pollutants that are commonly distributed in the same pattern and are toxic to humans. Plants are also able (in a limited capacity) to transport some of the mobile pollutants into roots and shoot via root. Depending on their chemical and physical properties, plants can draw the pollutants into the rhizosphere via the transpiration system. Within the rhizosphere the microbes degrade the pollutants for their metabolism, energy and cell growth (Harvey *et al*., 2002). The plants in turn supply oxygen and nutrients into the rhizosphere. Plant tolerance to pollutant correlates with ability to deposit in a bound residue fraction of plant cell walls and vacuole (Harvey *et al*., 2002).

9.2 Removal of phenol in soil is assisted by physical and biological factors

In the beginning of this research, the removal of phenol from soil in the presence of a plant was evaluated. The fate of phenol solution added to soil was different when added into soil with and without plants. However, the difference was frustratingly small, even when measured very close to the root surface. It was found that the initial concentration of phenol was reduced in soil, probably due to adsorption. Dilution and adsorption of phenol compound into the soil was quantified in order to make a correct estimate of the degradation process that occurs. Adsorption of phenol compounds occur depending on nature of soil characteristics, pH, and type of salts present in it (Gularte *et al*., 2014).

Similar experiment, using soil that was autoclaved, produced different results to those with soil that was not autoclaved. Adsorption was found to be higher in non-autoclaved soil (Chapter 3). Autoclaving is a common method used in degradation studies to differentiate between biotic (microbial processes) and abiotic reactions. Soil that has been autoclaved undergoes changes in chemical, physical and structural properties and inactivates microbes (Shaw *et al*., 1999; Berns *et al*., 2008; Baker and Mayfield, 1980). In autoclaved soil, there is a decrease in the aggregation of soil and an increase in dissolved organic matter (Berns *et al*., 2008), which in turn will affect the bioremediation process. This research finding also shows that the presence of microbial activity (non-autoclaved soil) seems to enhance phenol bioremedial activity. However, no further studies into microbial activity were carried out.

The rate of removal of different phenol compounds differ depending on the autoclaved and non-autoclaved soil. Phenol, ethylvanillin, DAHMP, vanillic acid and syringic acid degradation occurs rather slowly in autoclaved soil suggesting a possible involvement of microbes or enzymes present in the rhizosphere or soil. However, 3CP was not degraded in either conditions (autoclaved and non-autoclaved) in the tested soil. The results of this study indicates that removal of phenol from soil occurs both by non-biological factor, such as adsorption as well as microbes and plants (biological).

9.3 Accessibility to the underground root.

In the context of phytoremediation, the rhizosphere plays a vital role in influencing the plant root (Yamaga *et al*., 2010). This project aimed to investigate the effect plants had on phenol compounds in soil. This requires estimation and quantification of the chemicals in plant roots

in the rhizosphere that help in the removal of phenol. As the roots are located inside the soil (underground), it is not easy to access them for the purpose of sampling. Besides, soil is an extremely dynamic zone both spatially and temporally (Tandy *et al*., 2013). This can be overcome by using a microcosm system. This system enables sampling of soil solution and root in the rhizosphere to be easily accessible after the addition of phenolic compounds with minimal disturbance. Consequently, it was possible to evaluate the ability of rye and wheat plants to remove phenol compounds from the soil environment.

9.4 Phytoremedial activity occurs in zones close to the root

The zone of soil surrounding roots is affected by root uptake, exudation and deposition. In addition to the competition between the plant and microbes for nutrients. Therefore, it is a challenge to investigate the complex rhizosphere processes. It is further limited by difficulty in measurement and sampling techniques. A macro approach method (as described in section 2. 8.1) allows to investigate phenol removal in the rhizosphere using different plants. Both rye and wheat plants showed capability to remove phenolic compound from soil. Rye plants were found to have better ability to remove phenol compounds compared to wheat. Half-life of phenol compounds was shorter in the presence of rye plant in soil compared to wheat. However, only wheat plants were studied from there onwards (Chapter 4).

It is notable that microbes present in the rhizosphere do play a role in removal of phenol or other contaminants in suitable conditions (Masciandaro *et al*., 2013). In this research it was noted that a different phenol compounds are removed at different rates. Half-life of phenol compounds tested (phenol, 2CP and 3CP) was significantly lower in samples taken close to the roots compared to samples that was taken away from the root. This suggests the possible role of the rye and wheat plant root in removal of phenol. It is a known fact that plants can remove organic compound by direct uptake (De Araujo *et al*., 2002; Ucisik & Trapp,2006) or by producing exudates that promote growth of microbes that could utilize phenols as substrates (Zhou & Wu, 2013). The result show the ability to uptake phenol and syringic directly from the soil. However, rye removed phenolic compounds better than wheat. This type of study is vital for phytoremediation research and application, as it has been reported that even related native species may not react to contaminants in similar way. Besides, cultivars from same

species also are known to behave differently to contaminants (McCutcheon & Schnoor, 2004). Phenol appears to disturb the metabolic and growth processes in plants, and different plant species seems to have different tolerances levels to phenol (Flocco *et al*., 2002; González *et al*., 2013).

Recently, Shahsavari *et al*. (2013) investigate the ability of 11 species of plants to grow and survive in hydrocarbon-contaminated soil for further studies. They reported that maize and wheat showed the highest potential for use in phytoremediation based on emergence, shoot length, root length, and root/shoot biomass ratio production. Presence of both the plants also changed the structure of microbial community in the rhizosphere. The utilization of total petroleum hydrocarbon was significantly increased in presence of both maize and wheat from 57% in control soil to 72 and 66% in soil with maize and wheat respectively. Therefore, the authors selected wheat to investigate its potential to clean up the soil contaminated with polycyclic aromatic hydrocarbons (PAHs) including phenanthrene and pyrene (Shahsavari *et al.,* 2015). They reported that the presence of wheat increase the degradation rate of phenanthrene and pyrene in soil compared to the unplanted soil. The authors also reported that the numbers of microorganism in planted soils were higher than in soils without plant. These increased activities may be due to root exudates that released by root in to the soil such as organic acids, sugars, enzymes that stimulate the microbial growth (Shahsavari *et al.,* 2015).

Dams *et al*. (2007) studied the rhizoremediation of pentachlorophenol (PCP) using *S.chlorophenolicum* ATCC 39723 in soil in the presence of winter wheat (*T. aestivum*). The removal of PCP by *S.chlorophenolicum* was higher in the inoculated-planted soil than in the non-inoculated. The number of population of microbes increased in wheat rhizosphere compared to the soil without plant. The study demonstrated that wheat rhizosphere promotes the degradation of PCP. In addition, *S.chlorophenolicum* protect the plant growth from the phytotoxic effects. This effect was demonstrated by measuring plant weight and root length.

A micro approach (as described section 2.8.2) was applied in this study. This enabled the sampling of the soil solution and measurement of syringic acid in the rhizosphere at different distance from the root after the addition of syringic acid. In addition, it was possible to measure the uptake of syringic acid by the root using microcapillaries. The classical SiCSA technique was less suitable for phenol and some of its selected derivatives. This is because they readily

dissolve under paraffin oil, which is required for analysis of these compounds. The potential use of fluorocarbons was recognized too late in the program to be of use.

9.5 Differentiation of effects caused by microbes and plants

Phenol disappeared faster from soil after the addition in the presence of plant. Although the removal of phenol from soil was attributed partially to the phenolase in a slow rate compared to the rapid disappearance, the result reveal that phenolase activity was significantly higher from microcosms containing non-autoclaved soil with a plant (8%) compared similar microcosm without a plant (6.7%). In the same soil, in the absence of plants, a lower amount of phenol seems to be degraded. This confirms the role of the plant in removal activity of the phenol compounds tested. Sinsabaugh (2010) reported that plants could release extracellular phenol oxidase causing detoxification of phenolic compounds and enhancement of microbial activity. Some studies was reported the removal of phenol by enzymes that are released from plants (Dec & Bollag, 1994; Adler *et al*., 1994; Agostini *et al*., 2003; González *et al*.,2006).

However, we noted that phenol removal in soil by physical activity such as adsorption was happening faster compared to enzymatic activity. Many microbes have enzymes with the ability to convert phenolic compounds as a source of carbon and energy. Microorganisms are also able to mineralize phenols under aerobic conditions (Harvey *et al*., 2002). However, the microorganisms need to adapt to new habitats and synthesize enzymes to transform or degrade the chemical they come in contact with (Michalowicz & Duda, 2007). Recent studies have shown that numerous microorganisms are capable of degrading phenolic compounds. (Satchanska *et al*., 2015; Ahmad *et al*., 2015; Akbar, *et al*., 2015).

9.6 General physiological analysis of remaining compounds within different parts of plant

Wheat plants were shown to have the ability to remove phenol and syringic acid from soil by different mechanisms of phytoremediation. However, they seem to use different pathways of the removal process. Phenol is normally seen as a source of pollution whereas syringic acid is a naturally produced substance by plants. We found that phenol was predominantly removed by root uptake and partially transported to the leaf (Chapter 7). However, syringic acid was seen to be metabolized in roots itself. Wheat plants were able to clean up the soil by accumulating phenol in the leaf. Therefore, it may be possible to use it in phytoremediation.

Although it is suggested that most of the phenol was evaporated. This may depend on the amount of phenol and the level of pollution in the air.

9.7 Phytoremediation process in wheat

Truu *et al*. (2015) reported different strategies of phytoremediation to remove the contaminants from the soil. In this study, the phytoremediation of phenol was achieved using wheat plants by various of these strategies according to the result. Wheat was capable of removing phenol from soil by root uptake. This is called phytoimmobilization (Gao & Zhu, 2004) and is followed by transport to the leaf in a phytoextraction process (Reichenauer & Germida, 2008). After the uptake of phenol by the wheat root and followed by the transport to the leaf, two different pathways were suggested. Phenol was transported and concentrated in the leaf (phytoextraction/accumulation). In this situation the plant can be removed or harvested after remediation from the site (Susarla *et al*., 2002). However, this may depend on the level of the contaminants remaining in the plant. The other suggestion was that phenol might be transpiration through the stomata to the atmosphere and this may also be called phytovolatilization according to (Truu *et al*., 2015) despite the fact that phenol volatilization may either be safe or contribute to air pollution.

In addition, the result in this study indicates that some amount of phenol may be metabolized in the root. According to Reichenauer & Germida (2008), Ma & Burken (2003) and Eapen *et al*. (2007) organic contaminants can be metabolised by plant roots (phytoremediation) in three different stages. A transformation stage that is chemical modification of compounds such as oxidation to transform by the enzymes into more polar, water soluble form. The transformed compounds then conjugative with endogenous molecules such as sugar and peptide to make them less phytotoxic. This is followed by a compartmentalization stage. In this stage the contaminated compounds is transferred to different compartment of the plant cell such as the vacuole for storage.

The complex interaction between plant root and microbes to degrade the contaminants to a harmless from provides a different strategy of phytoremediation that is called rhizoremediation or rhizodegradation (Kuiper *et al*., 2004). The Chapter 3 result emphasize the influence of the rhizosphere on the rapid removal of phenol compared to bulk soil which is away from the root. Chapter 6 also shows that the degradation of phenol by phenolase extracted from wheat microcosm was higher than the soil microcosm itself. On the other hand, syringic acid as a biological compound compared to phenol was taken up by root and seemed to be metabolized

in the root without transport to the leaf. In addition, microbes play a role in the rapid removal of syringic acid from soil although it was significantly faster in the presence of plant.

Endophytic bacteria are characterized as non-pathogenic bacteria and they exist in the most of higher plant species. They are also have role to promote plant growth and pathogen control capabilities (Lodewyckx *et al.,* 2002). Some studies reported that the metabolic potential of their host plant can be supplement by endophytic bacteria through direct degradation (Barac, *et al.,* 2004; Germaine *et al.,* 2006). Therefore, the presence of such bacteria in plant contribute to improve phytoremediation processes (Afzal *et al.*, 2014). Although some achievement using plants alone has been reported in bioremediation, the combination of plants with microbes whether from rhizosphere or endophytic bacteria enhance potential for bioremediation (Truu *et al*., 2015).

9.8 Future work

This study presents more evidence that reinforces the findings that wheat and rye plants showed capability to remove phenolic compounds from soil and this could supply valuable information about the removal of phenols by plants. In wheat, it was demonstrated that wheat roots are able to remove phenol from soil and then the levels were reduced within the root tissue.

The result showed that phenol was removed through the plant in different pathways. Rapid degradation of phenol in the root, accumulation in leaf seem to be for long term and evaporation through the leaf to the air. This finding may lead to the use of these plants in the context of phytoremediation. Particularly in Saudi Arabia, where accidental spillage and industrial waste that lead to increased risks of environmental pollution are inevitable due to the wide spread petroleum industry. In addition to this, olives are a new crop in Saudi Arabia and the Al-Jouf region is the most important producer of olive oil for export (Hemida *et al*., 2014). Olive mill waste produced by this industry contributes to tremendous source of phenols accumulation in soil, in which they are disposed. The ever increasing production of olive oil, means that this industry has a significant role in soil contamination by phenols (El-Khateeb *et al*., 2014; Noubigh *et al*., 2009) such as vanillic acid and syringic acid (Noubigh *et al*., 2009). Wheat is the most important cereal crop, which consist of 29% of total agriculture area in Saudi Arabia. Al-Jouf is also a main region for wheat production in Saudi Arabia (Al-Qunaibet & Ghanem, 2014). These findings may contribute to other studies for optimization of phytoremediation process by wheat to maximize the removal of phenol. Also they will lead to the design of novel

transgenic plants with improved remediation traits. In order to fully exploit phytoremediation technology, the selection of suitable plant species is important for the remediation of specific compounds (Gonzalez *et al*.,2006). Plants found to be suitable for use in phytoremediation can be further enhanced in their capacity by cloning techniques. Genes responsible for certain pathways in metabolism can be identified and characterized and exploited. Role of microbes can be better understood and rhizosphere can be enriched for production of enzymes. Microbes contributing to the phytoremediation process in the rhizosphere can be identified and genetically modified in order to increase the capacity removal of the pollutants. Recent biotechnological advances have allowed the production of cheaper and more readily available enzymes through better isolation and purification procedures. Despite very little research having been done on the use of plant materials (roots, tissues, etc.) as an enzyme source, these could still be a good alternative due to its potentially lower cost (Duran & Esposito, 2000).

Rye was able to remove phenolic compounds faster wheat that indicate different plant has different capacity. This research finding supplied useful information for the use of the selected plants, which contain mechanism for an alternative cleanup processes in industries. Moreover, it could lead to more research into similar studies using other plants such as palms and olives that at are native or have ability to adapt and grow in Saudi Arabia.

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