Plant associated soil mechanisms of cadmium uptake and translocation in Chicory and Plantain

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Abstract

Cadmium (Cd) is a non-essential trace element that is extensively distributed in the environment. Cadmium is effectively absorbed by plant roots and transported to its aerial parts and plants growing in soils with high Cd concentration can accumulate Cd in their roots and shoots to levels which can threaten human and animal health. Elevated Cd concentrations in New Zealand agricultural soils are a function of the country's long-term history of using Cd-contaminated phosphate fertiliser. Recent studies have identified that two forage species chicory (*Cichorium intybus* L.) and plantain (*Plantago lanceolata* L.), which are increasingly used in New Zealand agriculture, accumulate a significantly higher shoot Cd concentration than traditional pasture species. The variation in Cd accumulation between forage species suggests that different plants have different abilities to absorb Cd in roots and translocate this trace element from roots to shoots. Thus, Cd uptake and the potential translocation of Cd to aerial tissues deserves more research, particularly for forage species of economic importance to countries such as New Zealand, where agriculture is dependent on pastoral grazing systems. Information from such studies will be useful in mitigating the continuing risk of Cd transfer into the food chain. The overall aim of this thesis is to better understand Cd uptake and translocation mechanisms in chicory and plantain.

Cadmium uptake by plant roots is a function of rhizosphere soil chemistry and the interaction between plant roots and soil solution. Plants exude Low Molecular Weight Organic Acids (LMWOA) into soil solution and these play a key role in regulating Cd bioavailability. A pot trial was conducted to evaluate the influence of increasing soil Cd concentration on the secretion of LMWOAs by chicory and plantain roots and to analyse their impact on plant Cd uptake. Chicory and plantain were grown under increasing Cd

levels and showed variable secretion of oxalic, fumaric, malic and acetic acids as a function of Cd treatment. Results revealed that the primary cause for the significant increase of shoot and root Cd concentration in both chicory and plantain, as a function of treatment level, is the significantly greater bioavailable Cd concentration in soil solution with increasing Cd treatment level. The significantly higher shoot Cd accumulation in chicory (18.63 mg Cd/kg DW) than plantain (4.22 mg Cd/kg DW) at the highest tested soil Cd concentration (1.6 mg Cd/kg) can be explained by increased acetic acid and reduced fumaric acid excretion from chicory relative to plantain.

Increased understanding of Cd translocation mechanisms in plants requires knowledge of the free Cd^{2+} ion concentration in xylem saps. However, the determination of low concentrations of free Cd^{2+} ions in a low volume of xylem sap poses an analytical challenge. To overcome this limitation, a thiosalicylic-acid-modified carbon-paste electrode was developed as an alternative and reliable measurement tool for the detection of free Cd^{2+} ions in environmental samples, including xylem saps. Compared to other Cd^{2+} ion ligands used to develop Cd^{2+}-ion-specific electrodes in literature, thiosalicylic acid is a readily available solid, which is stable to air, making it a conveniently handled ligand. The developed electrode showed a lower detection limit of 11 µg Cd/L (0.1×10^{-6} mol Cd/L) with a linear range from 20 to 100 µg Cd/L (0.18×10^{-6} to 0.88×10^{-6} mol Cd/L). To the best of my knowledge, this is the first time a Cd^{2+} ion-specific electrode was developed to determine free Cd^{2+} ion concentration in plant xylem sap. The modified electrode has the ability to distinguish between total Cd and free Cd^{2+} in solution and measure only the free Cd^{2+} ions in environmental samples, including xylem sap, with high precision (RSD<5%).

Subsequent analysis using the thiosalicylic acid modified electrode showed that Cd is mainly in a complex form in chicory and plantain xylem sap. Therefore, a glasshouse experiment was set up with six increasing Cd concentrations in hydroponic solution to assess the impact of LMWOA on xylem sap Cd translocation and shoot accumulation in chicory and plantain. Results revealed that both chicory and plantain showed variable production of oxalic, fumaric, citric, malic and acetic acids with increasing Cd concentration in the hydroponic media. The higher shoot Cd accumulation (by 28-208%) in chicory compared to plantain can be explained in terms of variations in LMWOA production between chicory and plantain. Functional relationship analysis showed that the primary cause for higher shoot Cd concentration in chicory relative to plantain is fumaric acid production in chicory xylem sap which may bind with Cd in chicory and translocate the metal towards shoots.

To explore the specific role of fumaric and acetic acids on Cd uptake and translocation in chicory, a glasshouse experiment was conducted with the external addition of fumaric and acetic acid into the hydroponic solution. Increasing fumaric acid concentration in the hydroponic solution showed the ability to reduce Cd uptake and translocation in chicory with a maximum reduction achieved at 10 mg/L and 50 mg/L fumaric acid treatment for root and shoot Cd accumulation, (respectively) for a solution concentration of 1 mg/L Cd. The shoot Cd concentration significantly increased at lower acetic acid treatment levels (1 mg/L) and reduced with increasing acetic acid concentrations from 10 mg/L to 50 mg/L in the presence of 1 mg Cd/L solution concentration. However, the root Cd accumulation increased as a function of acetic acid concentration in the hydroponic solution up to 50 mg/L acetic acid treatment. The root: shoot Cd concentration ratio showed a significant positive correlation ($R=0.729$ $P<0.05$) with acetic acid treatments (up to 50 mg/L treatment). Chicory biomass significantly reduced at all LMWOA treatments compared

to the control treatment in the presence of 1 mg Cd/L Cd level, showing that there was a limited potential ameliorative effect of LMWOA on Cd toxicity at any concentration for the experimental conditions used in this study.

This study highlights that variations in plant root LMWOA secretion and xylem sap LMWOA production between chicory and plantain can explain the different shoot Cd accumulation characteristics of these two forage species. This work shows that fumaric acid plays a fundamental role in both Cd uptake and translocation in chicory, while such a role is not clear for plantain. Low secretion of fumaric acid by roots and production of fumaric acid in chicory xylem sap aid to increase shoot Cd accumulation in chicory compared to plantain while low acetic acid secretion by chicory roots supports the high shoot Cd accumulation in chicory compared to plantain.

Future work is recommended to develop a new cultivar of chicory which express traits of variations in fumaric acid production and acetic acid production. Such work may yield new cultivars of chicory which restrict the translocation of Cd from roots to shoots in this important forage species. The future application of this work is to help develop strategies which could assist in mitigating high Cd accumulation in offal to maintain the standards of New Zealand's food production.

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List of Abbreviations

AAS	Atomic Absorption Spectroscopy
ASV	Anodic Stripping Voltammetry
ANZFA	Australia New Zealand Food Authority
Cd	Cadmium
CP	Carbon Paste
CV	Cyclic Voltammetry
DPASV	Differential Pulse Anodic Stripping Voltammetry
DW	Dry Weight
DM	Dry Matter
EDTA	Ethylenediaminetetraacetic acid
EPA	Environment protection Authority
FW	Fresh Weight
GFAAS	Graphite Furnace Atomic Absorption Spectroscopy
ICP AES	Inductively coupled plasma atomic emission spectroscopy
ICP	Inductively coupled plasma Mass spectroscopy
LMWOA	Low Molecular Weight Organic Acids
LSASV	Linear Sweep Anodic Stripping Voltammetry
MPL	Maximum Permissible Level
MRL	Maximum Residue Level
RSD	Relative Standard Deviation
SV	Stripping Voltammetry
SQWASV	Square-Wave Anodic Stripping Voltammetry
TEM	Transmission Electron Microscope
TF	Translocation Factor
TI	Tolerance Index
TSA-CP	Thiosalicylic Acid modified Carbon Paste

Chapter 1
Introduction

New Zealand's economy is heavily dependent on exports, and agriculture is a major contributor, with the country exporting agricultural products to more than 100 countries throughout the world. In 2018, around $31 billion in revenue was earned from dairy, meat and horticultural exports (Ministry of Primary Industries, 2019). These agricultural exports are increasingly marketed as high-quality products free from contamination (McLaughlin et al., 2000). However, to sustain production, New Zealand food production is highly dependent on soil fertility and soil management practices which have been shown to induce soil contamination. Therefore, the risk of contamination must be well managed (Loganathan et al., 2008; Schipper et al., 2011).

In New Zealand, cadmium (Cd) is a key environmental contaminant associated with the long-term high-rate application of phosphate (P) fertilizer, particularly on soils used for dairying and horticulture (Loganathan et al., 2003; Abraham, 2018). Even though the Cd concentration in P fertilisers used in New Zealand has been reduced to less than 280 mg Cd/kg P since 1997 (Salmanzadeh et al., 2016), many agricultural soils have been reported with elevated levels of soil Cd. For example, a soil survey conducted by Taylor (2007) reported that the mean soil Cd concentration of New Zealand agricultural soils (0.43 mg Cd/kg, n=825) is more than double that of non-agricultural soils (0.16 mg Cd/kg, n=372). Abraham (2018) conducted another soil survey and reported that the Waikato region, a major dairy and horticultural farming region, showed the highest mean total Cd concentration (0.85 mg Cd/kg).

Despite being a non-essential trace element, Cd can be absorbed by plant roots and transported to its aerial parts. Therefore, Cd contamination of New Zealand's most

versatile soils threatens to limit their use for high-value pasture and horticultural crops due to the risk of high Cd accumulation in plant tissues and subsequent trophic transfer along the food chain (Reiser et al., 2014). Cadmium mainly accumulates in animal's body tissues particularly in their kidneys and livers, and therefore, the New Zealand meat industry prevents the use of kidneys and livers from animals aged 30 months or older from human consumption, to minimize the risk of food standard maximum limit exceedances (Lee et al., 1994; Lee et al., 1996). However, studies have statistically modelled that animals younger than 30 months old grazing on Cd-enriched plants can accumulate Cd in kidneys and livers to above the maximum guideline levels of 2.5 (kidney) and 1.25 (liver) mg Cd/kg FW for human consumption (Reiser et al., 2014).

In 1990 new forage species were introduced to New Zealand's livestock grazing systems due to their high drought tolerance, nutrient content and environmental benefits. For example, chicory (*Cichorium intybus* L.) and plantain are (*Plantago lanceolata* L.) deeper rooting plants than perennial ryegrasses and could be useful in reducing nitrate-N leaching losses from ruminant grazed pasture systems (Li and Kemp, 2005; Woods et al., 2018). However, a study conducted by Stafford et al. (2016) showed that chicory and plantain can accumulate significantly higher Cd concentrations, from even low Cd soils, than grasses and legumes, which have traditionally been used in New Zealand. Studies suggest that different plant species have different abilities to absorb Cd from soils and translocate this element from root to shoot (Fu et al., 2018; Li et al., 2019b).

Many studies have shown that interactions between plant roots and soil in the rhizosphere, as a consequence of root-mediated changes in soil chemistry, can influence Cd bioavailability and that this plays a key role in Cd uptake by plant roots (Mench et al., 1991; Hinsinger et al., 2006). The rhizosphere is defined as the few

millimetres of soil surrounding the plant roots where numerous interactive processes occur, including root growth, respiration and nutrient uptake (Lux et al., 2010). Plant roots secrete many chemical compounds to the plant root-soil interface, and these can modify the physical and chemical characteristics of the rhizosphere and influence the chemical forms of contaminants in the soil such as Cd (Hill et al., 2002; Hinsinger et al., 2006). Many researchers have suggested that the rhizosphere soil receives Low Molecular Weight Organic Acid root exudates (LMWOA, e.g. malic, oxalic, acetic, fumaric and citric acids). These are negatively charged ions capable of complexing with bioavailable Cd^{2+} and modify plant Cd uptake (Han et al., 2006; Zhu et al., 2011).

Some root-to-shoot translocation studies that have investigated metal speciation in the xylem have indicated that metal translocation in xylem sap is mainly associated with LMWOAs (Senden and Wolterbeek, 1990; Cheng et al., 2016). However, other studies are in complete contrast, where they reported that Cd is mainly translocated as free Cd^{2+} ions in plant xylem sap without complexing with LMWOAs (Ueno et al., 2008; Hazama et al., 2015). Therefore, a better understanding of the mechanisms of Cd translocation for plant species important to agriculture, such as chicory and plantain, will be supported by knowledge of the forms of free and complexed Cd^{2+} ion concentration in xylem saps.

The identification and quantification of the LMWOAs in the xylem sap can be achieved using advanced separation techniques, such as gel exclusion and high-performance liquid chromatography. However, the quantification of low concentrations of free Cd^{2+} ions in a low volume of xylem sap poses a major analytical challenge. In recent years electrochemical methods such as stripping voltammetry (SV), which use selective working electrodes, have become promising tools to measure free metal ions (Ismail et al., 2019). Traditionally, the mercury hanging drop electrode has been used, however, its

application is now excluded from routine environmental analysis due to the toxic risk of mercury. In recent decades chemically modified carbon paste electrodes have attracted significant attention for trace metal analysis and further work to apply such electrodes to the environmental chemistry of Cd is an opportunity for novel research. Therefore, the development of a chemically modified carbon-paste working electrode to quantify free Cd^{2+} ions in the plant xylem sap has attracted much attention due to the scope for innovation.

Overall, the impact of elevated levels of Cd in New Zealand agricultural soil on Cd uptake and the potential translocation of Cd into important forage species such as chicory and plantain deserves more research. Thus, the rationale for this thesis research is to better understand the mechanisms of Cd uptake and translocation associated with LMWOAs in Cd-accumulating forage species, as such information will be useful in mitigating the continuing risk of Cd transfer into the food chain. The work in this PhD thesis is designed to help develop the data sets necessary to fill these existing knowledge gaps.

1.1 Research focus

- To determine the effect of Cd concentration in growth media on the type and quantity of LMWOA secretion by chicory and plantain roots and their influence on plant Cd uptake.
- To develop a Cd^{2+} ion-specific electrode to quantify low concentrations of free Cd^{2+} ions in xylem saps.
- To determine the variations in composition and concentration of LMWOA production in chicory and plantain xylem sap as a function of Cd concentration in the growth media and their influence on Cd translocation.
- To investigate the effect of exogenous LMWOA on the shoot and root Cd variations in chicory.

1.2 Thesis Structure

This thesis is organised into seven chapters. Chapter 1 introduces the topic of interest, focusing on the overall context of plant Cd uptake by roots and xylem sap translocation to shoots. Chapter 2 reviews the knowledge relating to soil and plant factors affecting the Cd bioavailability in soil and the general mechanisms involved in plant Cd uptake and translocation by different plants. In addition, Chapter 2 presents detailed information on the analytical methods used to detect different Cd species in xylem saps as well as the current state of knowledge with regard to Cd in New Zealand agriculture. Chapter 3 describes the effect of soil Cd on LMWOA secretion by plant roots and the influence on plant Cd uptake by chicory and plantain. Chapter 4 describes the development of a Cd ion-specific electrode modified with thiosalicylic acid to determine the free Cd^{2+} ion concentration in soil solutions and xylem saps. Chapter 5 explains the consequence of Cd in growth media on LMWOA production in the xylem sap of chicory and plantain. Further, Chapter 5 analyses the influence of produced LMWOAs on Cd translocation via xylem sap in chicory and plantain. Chapter 6 describes the effect of several exogenous LMOWOAs (which were identified from the previous chapters) on Cd uptake by chicory. Chapter 7 summarises the key findings from the research chapters and explains the application of the knowledge developed in this thesis to develop strategies which could assist in avoiding high Cd accumulation in offal to maintain the standards of New Zealand's food production.

Chapter 2
Literature Review

2.1 Introduction

Cadmium (Cd) is a key environmental contaminant associated with long-term high application rates of superphosphate fertiliser to soils used for dairying and horticulture. Although Cd is considered to be a non-essential element for plants, it is effectively taken up by the root systems of many plant species and can be subsequently transported throughout the plant. Recent studies indicate that elevated levels of Cd in New Zealand soils can lead to a Cd concentration in forage species such as chicory (*Cichorium intybus* L.) and plantain (*Plantago lanceolata* L.) that is orders of magnitude higher than in ryegrass or clover. Results of such studies suggest the different abilities of pastoral species used in New Zealand to both absorb Cd from soils and to translocate the metal from roots to shoots. However, there have been no studies published on the Cd uptake mechanisms of common forage species used in New Zealand agriculture and it is within the context of this knowledge gap that the research described in this doctoral thesis has been undertaken. This literature presents a summary of the current state of knowledge with regard to Cd in New Zealand agriculture and general mechanisms involved in plant Cd uptake and translocation by different plants.

2.2 Cadmium: origin and toxicity

Trace elements (TE) are naturally present throughout the environment (Kabata-Pendias and Pendias, 1992) and can be either essential for plant and human growth such as iron, zinc, copper, manganese, and cobalt, or non-essential such as cadmium and lead (Hooda,

2010). Cadmium naturally occurs with a background concentration of about 0.1 mg/kg in the earth's crust (Tchounwou et al., 2012) although anthropogenic activities such as mining, fossil fuel combustion, sewage sludge disposal and the addition of phosphate (P) fertiliser to agricultural land have elevated the Cd concentrations in soil (Alloway and Steinnes, 1999). Despite being a non-essential trace element, Cd can be taken up by plant roots, translocated to aerial parts, accumulated in shoot tissues, and can present an ingestion risk for both animals and people (Ubeynarayana et al., 2021) (Figure 2.1).

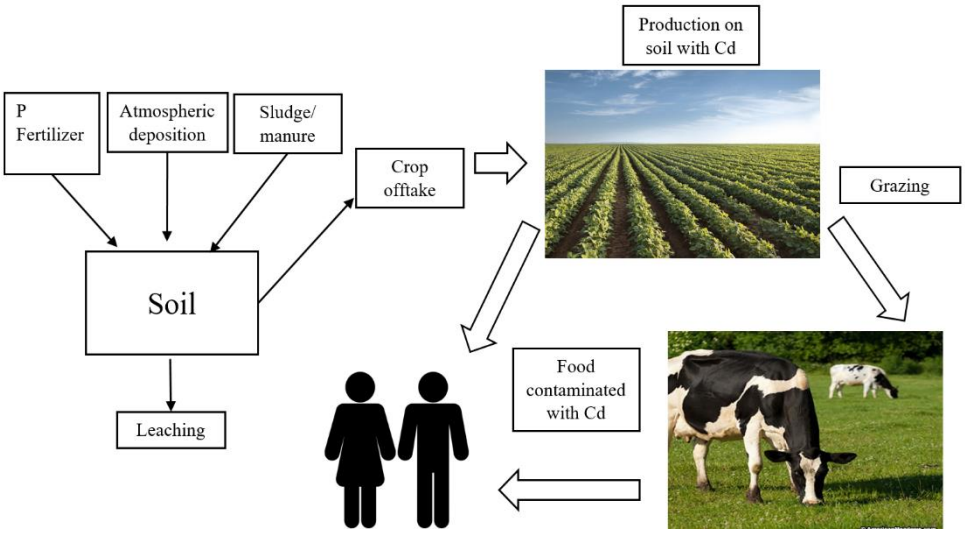

Figure 2.1. The Cd input-output balance in agricultural soils and the risk of contamination through the food chain (redrawn and adapted from (Smolders, 2013)).

Cadmium uptake by plants is affected by a number of factors related to changes in key soil properties (soil particle size, pH, temperature, cation exchange capacity), particularly in the soil attached to roots (rhizosphere), and plant physiology characteristics (root surface area, root exudation) (Lux et al., 2010). Cadmium accumulation in plant tissues and subsequent trophic transfer along the food chain presents a risk to the environment, food quality, and human health (Smolders, 2013) (Figure 2.1). Retention of Cd in human

or animal bodies for many years may lead to cancer, bone lesions, lung insufficiency, teratogenic effects, renal disturbances, anaemia, hypertension and weight loss. It is therefore classified as a potential human carcinogen (group 2B) by the US Environmental Protection Agency (EPA), and a human carcinogen (group 1) by the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) (Godt et al., 2006).

2.3 Cadmium sources and levels in the soil

Cadmium accumulation in the soil can occur due to both natural and human-derived sources. Cadmium is a naturally occurring contaminant in the phosphate rocks used to manufacture phosphate fertiliser, thus, soil Cd concentrations largely depend on parent rock material (Roberts et al., 1994). Cadmium in sedimentary phosphate rocks is present at a higher concentration and is more reactive than Cd present in igneous rocks (Table 2.1). The lowest soil Cd concentration (0.1 to 0.3 mg Cd/kg) has been recorded in soil derived from igneous rock, while the highest soil Cd concentration (0.3 to 11 mg Cd/kg soil) has been reported in soils derived from sedimentary rocks (McLaughlin and Singh, 1999).

Table 2.1. Average Cd concentration in different phosphate rocks (PR) (McLaughlin and Singh, 1999; Loganathan et al., 2003).

Sedimentary PR	**Cd (mg/kg)**	**Igneous PR**	**Cd (mg/kg)**
Gasfa	38	Kola	0.2
Morocco (Boucraa)	38	Chatham Rise	2
North Carolina	41	North Florida	3
Christmas Island	43	Phalaborwa	4
Toga	51	Jordan	5
Ocean Island	99	Sechura	11
Nauru	100	Arad	12

Human activities such as mining, fossil fuel combustion, sewage sludge disposal and the addition of phosphate (P) fertiliser to soil elevate the Cd concentration in soil (Schipper et al., 2011). As a result, land use category can be considered as a key driver for topsoil Cd accumulation; a summary of previous studies conducted for Cd accumulation in various agricultural land uses in different countries are listed in Table 2.2. Generally, pasture and horticulture land-uses show higher Cd concentrations in soil than non-cultivated soil. The reason for this is likely to be the long-term application of phosphate fertiliser in most pastoral and horticultural soils (Taylor, 2007; Yan et al., 2015). In Australia, Jinadasa et al. (1997) reported that the mean Cd concentration for 29 agricultural topsoil samples (0-150 mm) ranged from 0.11 to 6.37 mg Cd/kg (mean Cd concentration 1.33 mg Cd/kg), while the mean Cd concentration in topsoil collected from an uncropped area was 0.36 mg Cd/kg. A soil survey using data collected from 486 studies into the Cd concentration in Chinese arable soil showed that the average soil Cd concentration in arable land of China was 0.27 mg Cd/kg (Zhang et al., 2015). A study done by Sanderson et al. (2019) found that the soil Cd concentration in potato cultivating farms (20 farms) in Jamaica ranged between 0.1-62.3 mg Cd/kg.

Table 2.2. Studies on mean soil Cd concentration as a function of land use in different countries.

Country	**Land use type**	**Mean Cd concentration (mg Cd/kg)**	**Reference**
Australia	Agricultural	1.33	Jinadasa et al. (1997)
Netherlands	Horticulture /Agriculture	0.50	Wiersma et al. (1986)
Canada	Orchards	0.56	Frank et al. (1976)
United States	Agricultural	0.13	Holmgren et al. (1993)
New Zealand	Pasture	0.44	Roberts et al. (1994)
China	Arable soil	0.27	Zhang et al. (2015)
Spain	Agricultural	0.30	Pérez-Sirvent et al. (2009)

2.4 Cd accumulation in New Zealand agricultural soils

Several studies have conducted research on the natural background Cd concentration in New Zealand and reported this as 0.20 mg Cd/kg (Roberts et al., 1994), 0.16 mg Cd/ kg (Taylor, 2007) and 0.20 mg Cd/kg (Longhurst et al., 2004), with elevated levels of Cd, measured in New Zealand agricultural soils consistently associated with the long-term application of phosphate fertiliser (Loganathan et al., 2003; Abraham, 2018). Historically, the primary source of New Zealand's phosphate fertiliser was phosphate rock imported from the Pacific Island of Nauru (Table 2.1). The superphosphate fertilizers produced from Nauru rock-phosphate is now known to be the most Cd contaminated (450 mg Cd/kg P) in the world. In recognition of this issue, and the realisation that Cd in fertiliser was leading to elevated soil Cd concentrations in agricultural and horticultural lands, the New Zealand fertilizer industry voluntarily limited the Cd concentration in New Zealand P fertiliser to 280 mg Cd/kg P from 1997 (Abraham, 2018).

Cadmium accumulation in New Zealand agricultural soils varies from region to region based on the phosphate fertiliser use history and land use type (Taylor, 2007; Stafford et al., 2018). Table 2.3 summarises the findings of recent studies which provide an overview of the range and variability in national soil Cd concentrations based on land use, region and different phosphate fertiliser history. Cavanagh (2014b) reported that the highest median Cd concentrations were observed in Waikato, Taranaki and Bay of Plenty regions (0.74, 0.71 and 0.54 mg Cd/kg, respectively) which are major dairy and horticultural farming regions. This is in agreement with the early findings of Taylor (2007) who assessed the soil Cd concentration in soils from different New Zealand regions and showed that the highest concentrations of Cd were in Taranaki (0.66 mg Cd/kg) followed by Waikato (0.60 mg Cd/kg) and Bay of Plenty (0.52 mg Cd/ kg) (Figure 2.2). The

productive soils in these areas are developed from volcanic materials, where allophane, an Al-rich mineral showing large surface area and pH-dependent charge characteristics, is present and contributes to increased P sorption and subsequent Cd accumulation (Taylor, 2007).

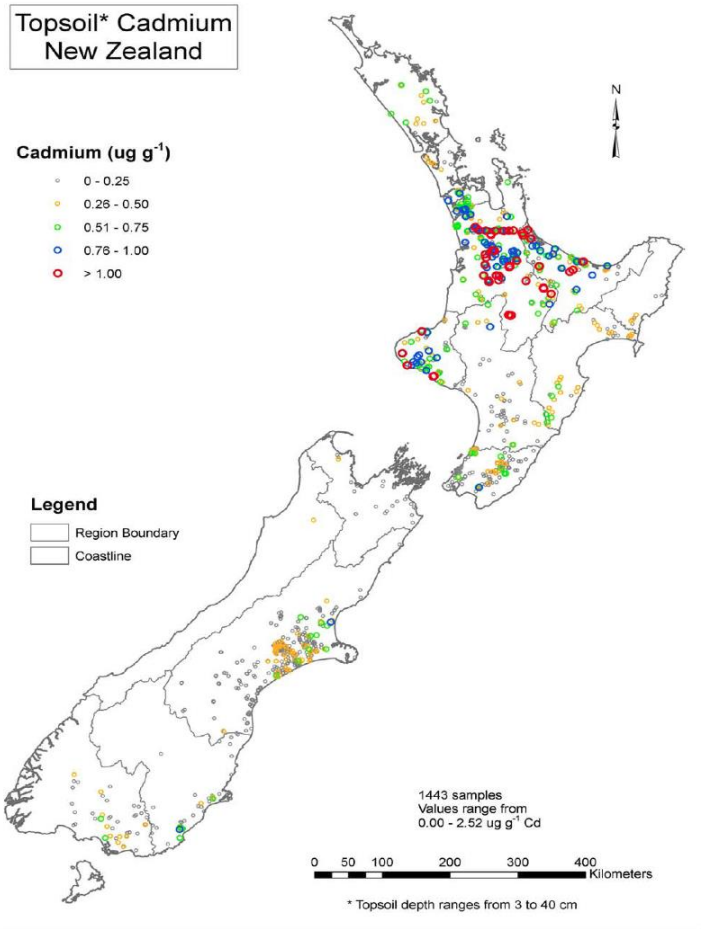

Figure 2.2. Cd accumulation in New Zealand based on regions (Taylor, 2007).

Roberts et al. (1994) carried out a national survey on Cd accumulation in 312 pastoral sites in New Zealand and reported that the mean Cd concentration for pastoral soil (0-75mm depth) was 0.4 mg Cd/kg. McDowell et al. (2013) surveyed 939 agricultural soil samples and 289 non-agricultural soil samples at 0-100 mm depth across New Zealand. They reported the mean Cd concentration in agricultural soil as 0.32 mg Cd/kg

while for non-agricultural soil the value was 0.14 mg Cd/kg (Table 2.3). Martin et al. (2017) analysed the trace metal and metalloid (As, Cd, Cr, Cu, Hg, Ni, Pb, Zn) concentrations in 284 topsoil (0-300 mm) samples from agriculture and non-agriculture soils in southern New Zealand. They found that the topsoil Cd concentration for pasture lands ranged from 0.01 to 1.31 mg Cd/kg, while the average Cd concentration for natural background soil ranged from 0.01 to 0.24 mg Cd/kg. Wakelin et al. (2016) collected soil samples from 26 pasture sites (0-75 mm depth) and showed the mean soil Cd concentration to be 0.23 mg Cd/kg. Longhurst et al. (2004) conducted a survey on a total of 398 sites covering both farmed pastoral and non-farmed sites in the major provinces in New Zealand and found that the mean Cd concentration in farmed soil was 0.44 mg Cd/kg and the mean Cd concentration in non-farmed soil was 0.20 mg Cd/kg. Stafford et al. (2018) analysed the soil Cd concentration in two long-term dairy farms with different phosphorus fertiliser application histories in the Waikato and Canterbury regions. They observed a higher soil Cd concentration (0-150 mm depth) in the Waikato (Allophanic soil) farm (mean: 1.04 mg Cd/kg, range: 0.48-1.64 mg Cd/kg) than the Canterbury (Gley soil) farm (mean: 0.34 mg Cd/kg, range: 0.15-0.64 mg Cd/kg). Cavanagh (2014b) found that land use has an effect on the Cd concentration of soil as a function of differential rates of fertiliser. They found that the mean soil Cd concentration by land use decreased in order of; dairying (0.59 mg Cd/ kg)> orchard (0.55 mg Cd/ kg)> dry stock (0.33 mg Cd/kg)> arable (0.28 mg Cd/kg)> non-agricultural (0.13 mg Cd/kg).

Table 2.3. Summary of studies investigating Cd concentrations in New Zealand soils.

Details	Findings	Reference
Cd concentration measured in soil samples from pasture sites including high-intensity dairy production units to low-intensity hill country grazing units.	• The background soil Cd concentration ranged from 0.06-0.85 mg Cd/kg with a mean of 0.23 mg Cd/kg soil.	Wakelin et al. (2016)
A survey conducted to evaluate the soil Cd concentration in agricultural and non-agricultural soil.	• The mean Cd concentration in agricultural soil was 0.32 mg Cd/kg. • The mean Cd concentration in non-agricultural soil was 0.14 mg Cd/kg.	McDowell et al. (2013)
A survey conducted in 312 pastoral farming sites to compare the Cd concentration in native and pastoral soil.	• The mean Cd concentration in native soil was 0.20 mg Cd/kg. • The mean Cd concentration in pastoral soil was 0.44 mg Cd/kg.	(Roberts et al., 1994)
A survey conducted on soil Cd variations in farmed and non-farmed sites in major provinces in New Zealand.	• The mean Cd concentration in farmed soil was 0.44 mg Cd/kg. • The mean Cd concentration in non-farmed soil was 0.20 mg Cd/kg.	Longhurst et al. (2004)
Cd concentration measured in 241 pasture soil and 43 natural background from southern New Zealand.	• The soil Cd concentration in the pasture was ranged from 0.005 to 1.31 mg Cd/kg. • The soil Cd concentration natural background soil was ranged from 0.005 to 0.24 mg Cd/kg.	Martin et al. (2017)
Assessed the soil Cd concentration from different regions and land use categories throughout the New Zealand.	• Taranaki region showed the highest total soil Cd concentration (0.66 mg Cd/kg) followed by Waikato (0.60 mg Cd/kg) and Bay of plenty (0.52 mg Cd/kg) regions. • The dairying land use category showed the highest national average for Cd concentration (0.73 mg Cd/kg). • Sheep land use category showed the lowest national average for Cd concentration (0.33 mg Cd/kg).	Taylor (2007)
Assessed the soil Cd concentrations in different regions in New Zealand.	• Waikato showed the highest median Cd concentration of 0.74 mg Cd/kg followed by Taranaki (0.71 mg Cd/kg) and Bay of Plenty (0.54 mg Cd/kg) areas.	Cavanagh et al. (2014b)
A soil survey conducted to assess the soil Cd concentration from different regions and land use categories in New Zealand.	• Waikato region showed the highest total soil Cd concentration (0.85 mg Cd/kg) in the country. • Otago had the lowest soil Cd concentration (0.2 mg Cd/kg) in the country. • Dairy was the land use with the highest Cd concentration (0.6 mg Cd/kg) followed by sheep (0.4 mg Cd/kg) and beef, and cropping (0.35 mg Cd/kg).	Abraham (2018)
Two long term dairy farms in Waikato (Allophanic) and Canterbury (Gley) regions used to determine the variations in soil Cd.	• The mean Cd concentration in Waikato farm was 1.04 mg Cd/kg. • The mean Cd concentration in Canterbury farm was 0.34 mg Cd/kg.	Stafford et al. (2018)
Assessed the variation in Cd concentration between different soil types mainly collected from pasture sites.	• The total Cd concentration in orthic Allophanic soil showed the highest Cd concentration (1.34 mg Cd/kg). • Orthic brown soil showed the lowest Cd concentration 0.04 mg Cd/kg.	Gray et al. (2000)

Taylor (2007) also reported that dairying has the highest national average for Cd concentration for grazing land (0.73 mg Cd/kg) followed by deer (0.68 mg Cd/kg), horse (0.53 mg Cd/kg), beef (0.42 mg Cd/kg) and sheep (0.33 mg Cd/kg). Abraham (2018) analysed the soil Cd data collected by the fertiliser industry and regional councils in New Zealand between the years 2006 to 2015. The author reported that that dairy was the land use with the highest Cd concentration (0.6 mg Cd/kg) followed by sheep (0.4 mg Cd/kg) and beef and cropping (0.35 mg Cd/kg).

2.5 Cd accumulation in forage and food species

Variations of Cd absorption and accumulation across plants depends on the physiological characters of species and cultivar (Bingham et al., 1975). Generally, species within the Solanaceae (e.g.potato), Asteraceae (e.g. lettuce, chicory) and Brassicaceae (e.g. turnip, kale, swede) families accumulate a higher Cd concentration than the Gramineae (e.g. ryegrass, wheat) and Leguminosae (e.g. white clover) families (Kuboi et al., 1986). The uptake of Cd across economically important species in New Zealand was shown to decrease in the order of leafy vegetables> root vegetables> grain crops> fruit (Gray et al., 1999). Gray et al. (1999) found that plant Cd concentrations decreased in the order lettuce (*Lactuca sativa*)> carrot tops (*Daucus carota subsp. Sativus*)> carrot root> lucerne (*Medicago sativa*)> cabbage (*Brassica oleracea var. capitata*)> wheat (*Triticum*)> maize (*Zea mays*)> ryegrass (*Lolium*)> clover (*Trifolium*)> barley (*Hordeum vulgare*). In a study, the Cd uptake by cultivars of wheat, potatoes (*Solanum tuberosum*), onions (*Allium cepa*), leafy green vegetables, and important pasture and forage species were assessed at twenty commercial field locations. The results showed that tissue Cd accumulation was highly dependent on the cultivar (Cavanagh et al., 2016).

To minimise potential risks associated with excessive Cd intake via consumption of food crops, maximum permissible limits (MPL) of Cd in edible plant parts have been established by the Codex Alimentarius Commission of the Food and Agriculture Organization (FAO), and Australia and New Zealand Food Regulation Authority (ANZFA) (FSANZ, 2013; CODEX, 2018) (Table 2.4). However, the ANZFA guidelines are stricter for some food crops compared to Codex guidelines which support maintaining high food standards.

Table 2.4. MPL values for Cd in edible plant parts (FSANZ, 2013; CODEX, 2018).

Edible plant tissue	**CODEX level for Cd (mg/kg Fresh Weight (FW))**	**ANZFA level for Cd (mg/kg Fresh Weight (FW)) (After 2002)**
Brassica vegetables	0.05	
Fruiting vegetables	0.05	
Leafy vegetables	0.2	0.1
Legume, cereals, potato	0.1	0.1
Root and tuber vegetables	0.1	0.1
Wheat	0.2	0.1
Rice	0.4	

Table 2.5 shows a summary of recent studies conducted on variations of Cd accumulation in different food crops grown in New Zealand. Roberts et al. (1995) reported tissue Cd concentrations of 0.07, 0.04, 0.02 and 0.02 mg Cd/kg FW in wheat grain, lettuce, potato, and onion, respectively. While the mean Cd concentration in wheat grain was lower than the MPL of Australia and New Zealand Food Authority (ANZFA), the grain Cd concentration ranged from 0.02 to 0.19 mg Cd/kg; some reported concentrations were higher than MPL for some locations. Gray et al. (2019b) conducted a survey to determine the grain Cd concentration in wheat grown across 34 sites in New Zealand including sites in both the North (6 sites) and South (28 sites) islands in 2016-2017. They reported that the overall mean concentration in wheat grain (0.07 mg Cd/kg FW) was below the MPL of 0.1 mg Cd/kg FW, but 7% of grain samples exceeded the MPL (Table 2.5). Cavanagh

et al. (2019) conducted a field survey on the variations of Cd concentration of onion, lettuce and spinach grown in several fields across New Zealand in the years 2016 and 2017. They found that the mean Cd concentration of onion and spinach was 0.02 and 0.06 mg Cd/kg, respectively. However, the authors found that the mean concentration in spinach at several sites exceeded the ANZFA MPL for Cd in leafy greens of 0.1 mg Cd/kg.

Table 2.5. Summary of relevant studies investigating Cd accumulation in food crops grown in New Zealand.

Details	Findings	Reference
A survey conducted to determine the Cd concentration in wheat cultivars across seven sites in New Zealand, between 2016-2017.	• The wheat Cd concentration ranged from 0.01-0.21 mg Cd/kg FW with a mean Cd concentration of 0.07 mg Cd/kg FW. • Only 7% of wheat grain samples exceed the MPL value of 0.1 mg Cd/kg FW.	Gray et al. (2019b)
Cd concentration measured in ten potato cultivars grown in three field sites in New Zealand.	• The overall Cd concentration in all potato cultivars ranged from 0.04 to 0.28 mg Cd/kg DW with a mean Cd concentration of 0.12 mg Cd/kg DW.	Gray et al. (2019a)
Cd concentration measured in spinach, onion, potato and wheat which collected from 48 sites across New Zealand.	• Spinach (*Spinacia oleracea*) showed the highest Cd concentration (1.00 mg Cd/kg) followed by onion (0.20 mg Cd/kg), potato (0.19 mg Cd/kg) and wheat (0.10 mg Cd/kg).	Yi et al. (2020)
A field survey conducted to determine the variations in Cd concentration of onion, and spinach in the years 2016 and 2017.	• The mean Cd concentration of onion was 0.02 mg Cd/kg (range: 0.01-0.05 mg Cd/kg). • The mean Cd concentration of spinach was 0.06 mg Cd/kg (range: 0.01-0.10 mg Cd/kg).	Cavanagh et al. (2019)
A survey conducted to evaluate the Cd accumulation in different food plant species grown in Auckland market gardens and Canterbury wheat farms.	• Wheat showed the highest tissue Cd concentration (mean: 0.07 mg Cd/kg FW, range: 0.02-0.19 mg Cd/kg FW). • Onion showed the lowest Cd concentration (mean: 0.02 mg Cd/kg FW, range: 0.01-0.11 mg Cd/kg FW).	Roberts et al. (1995)

2.5.1 Cadmium uptake variations in New Zealand livestock grazing forage plants

Since the 1990s, a variety of new forage species have been introduced to New Zealand livestock grazing systems due to their high drought tolerance and high nutrient quality.

For example, summer- and winter-grazed forage brassicas are introduced as a source of high-quality feed for livestock when pasture feed quality is poor. In addition, chicory (*Cichorium intybus* L.) and plantain (*Plantago lanceolata* L.) are increasingly being sown as monoculture stands or in combination with red or white clover, due to their high nutrient quality, growth rate and persistence over the hot summer period. Further, they are deeper rooting plants than perennial ryegrasses, which could be useful to reduce nitrate-N leaching losses from grazed pasture systems (Li and Kemp, 2005; Woods et al., 2018).

Little data exists on Cd accumulation in forage species. Parker et al. (2008) reported that the pasture Cd concentration was 4-5 times higher in a plantain/ chicory/ red clover/ white clover forage stand (0.36-0.75 mg Cd/kg DM) than ryegrass/ white clover pasture stand (0.11-0.22 mg Cd/kg DM). Stafford et al. (2016) conducted a greenhouse trial involving 12 forage species and showed that the mean tissue Cd concentration decreased in the order of chicory> plantain> turnip (*Brassica rapa subsp. rapa*)> lucerne> sheep's burnet> strawberry clover> kale (*Brassica oleracea*)> perennial ryegrass (*Lolium*)> hares foot trefoil> red clover> crimson clover> white clover. Chicory and plantain had significantly greater mean tissue Cd concentrations (1.6 and 0.7 mg Cd/kg DM, respectively) than all other species (Figure 2.3). This observation built on an earlier field study by Martin et al. (1996) who reported that even at extremely low soil total Cd concentrations (0.004-0.020 mg Cd/kg) chicory was able to accumulate high leaf Cd concentrations (1.6-2.4 mg Cd/kg DM). Crush et al. (2019) reported that the mean foliar Cd concentration in high Cd uptake cultivars and low Cd cultivars of chicory was 15.1 mg Cd/kg and 8.1 mg Cd/kg, respectively, when grown on in Horotiu silt loam soil with a total soil concentration of 1.2 mg Cd/kg.

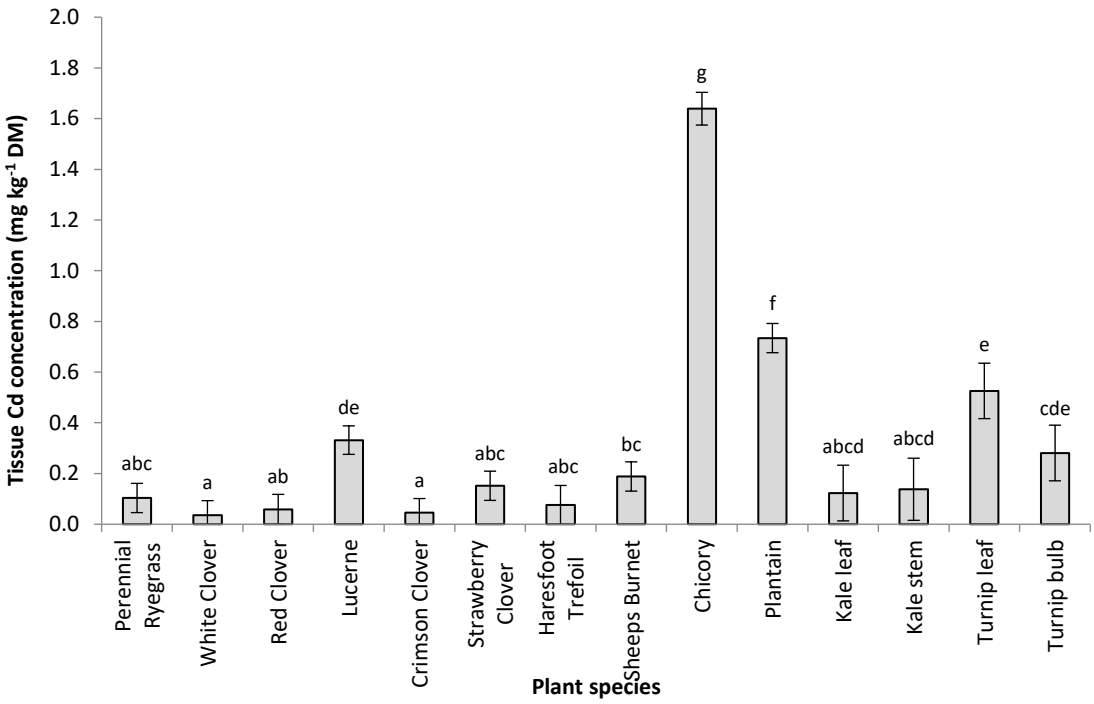

Figure 2.3. Mean tissue Cd concentration of various forage species used in New Zealand farming systems (redrawn and adapted from Stafford et al. (2016)).

2.6 Cadmium accumulation in grazing animals

The amount of Cd taken up by grazing animals will primarily depend on the level of Cd they are exposed to through the pasture (Roberts et al., 1994). Cadmium can accumulate in an animal's body tissues, particularly in their kidney and liver as animals produce a metal-binding protein called metallothionein (Lee et al., 1996). Since the Cd is accumulated throughout the animal life, kidneys and livers will show higher Cd concentrations depending on the age of the animal. Therefore, the New Zealand meat industry began to discard kidneys and livers from animals aged 30 months or older, to minimise the risk of food standard maximum limit exceedances (Lee et al., 1994). Prior to 2003, the Maximum Residue Level (MRL) for the Cd content of meat and offal for human consumption was 1mg Cd/kg FW (Roberts et al., 1994). Roberts et al. (1994) found that about 22-28% of sheep and 14-20% of cattle kidney's in New Zealand

exceeded the MRL from 1989-1991. Solly et al. (1981) observed that the Cd concentration in the kidney exceeded the MRL in about 5% of sheep, cattle and pig. Even 22% of young sheep (<30 months age) were reported by Lee et al. (1994) to have a kidney Cd concentration that exceeded the 1 mg Cd/kg limit (Table 2.6). Strategies to minimise animal grazing on Cd-rich forages which can manage Cd accumulation in grazing livestock are, therefore, of critical importance to the ongoing sustainability of New Zealand pastoral farming systems (Lee et al., 1994; Lee et al., 1996; Stafford et al., 2016).

The maximum Cd residue level in New Zealand meat changed in December 2002 with the permissible level increasing for kidney and liver but decreasing for meat flesh (Table 2.6). These changes in MRL were implemented as 'The Australia and New Zealand Food Authority (ANZFA)' formed as a new joint organisation. A study by Reiser et al. (2014) showed that 2.9% of cows' kidney, and 1.4% and 2.9% of sheep's kidneys and livers, had a Cd concentration which exceeded the maximum permitted value. A study by Stafford et al. (2016) statistically modelled a lamb feed intake of 1 kg DM of chicory (1.64 mg Cd/kg DM) per day for 60 days and predicted a kidney Cd concentration of 1.45 mg Cd/kg FW. This author reported that even though the predicted kidney Cd concentration for lambs grazing a pure chicory stand did not exceed the current New Zealand MPL (FSANZ, 2013), it exceeded the current European Commission (EC) food standard MPL of 1.0 mg C/kg FW (EC, 2014).

Table 2.6. The maximum residue level of Cd in New Zealand meat food (Loganathan et al., 2008; EC,2014: CODEX, 2018).

Organ	**ANZFA Before Dec. 2002 (mg Cd/kg FW)**	**ANZFA After Dec. 2002 (mg Cd/kg FW)**	**EC (mg Cd/kg FW)**	**CODEX (mg Cd/kg FW)**
Kidneys	1.0	2.50	1.0	0.2
Liver	1.0	1.25	1.0	0.2
Meat Flesh	1.0	0.05	-	0.1

2.7 The Fate of Cd in soil

The source of Cd added to New Zealand pastoral soils is new Cd added through fertilisation (Figure 2.1, Table 2.3), or recycled Cd returned to soil with animal excrement or plant residues. Once Cd is added to the topsoil, the element will undergo a series of reactions, such as direct sorption to soil colloids, reaction with inorganic and organic ligands and subsequent adsorption of the ligand complexes to soil colloids, or precipitation as a compound with varying solubility. A small fraction of Cd in soil remains as the free Cd^{2+} ion (Figure 2.4) (Loganathan, 2012).

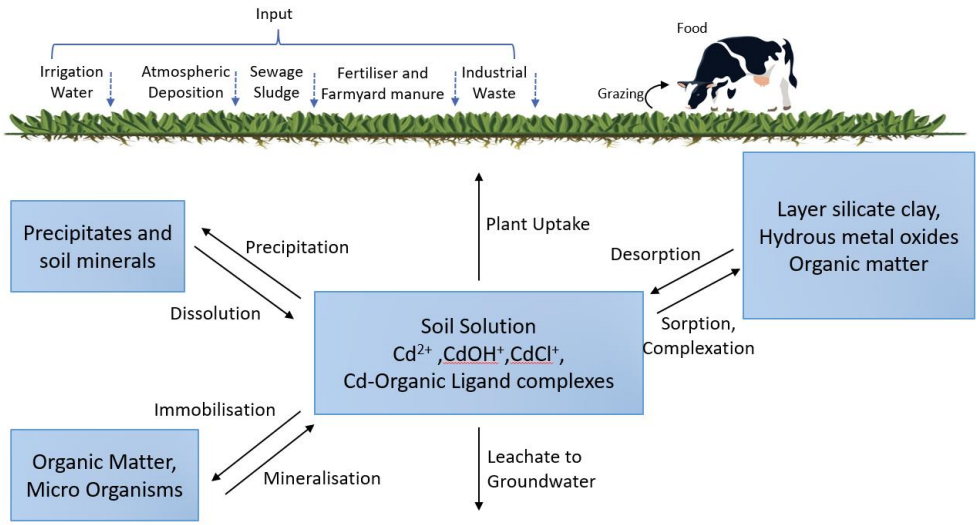

Figure 2.4. The inputs outputs and dynamics of Cd in soil (redrawn and adapted from Loganathan (2012)).

In order to estimate the ecological risk associated with Cd contamination in soil, it is necessary to understand the bioavailability of Cd in the soil. The bioavailability of soil elements can be defined as the potential soil fraction of an element that can be transferred from soil to living organisms (Jeyakumar et al., 2010). Romić et al. (2014) defined the soil exchangeable and water-soluble fractions of trace metals as bioavailable fractions. However, the most bioavailable soil Cd species to plants is free Cd^{2+} ions in soil solution (Geebelen et al., 2003). The speciation of Cd in the soil can be operationally described by the distribution of Cd among defined chemical species within the soil system (Yang et al., 2019). The chemical species in the soil solid- and solution-phase can be categorised as follows (Hooda, 2010);

- Exchangeable/ water-soluble fraction (bioavailable fraction)
- Acid soluble fraction (carbonates)
- Adsorbed or occluded in hydrated oxides of iron and manganese (reducible)
- Complexed or occluded within organic matter (oxidizable)
- Residual fraction

Many studies have been conducted to determine Cd speciation in soil and land use types (Gray et al., 2000; Renella et al., 2004; Xin et al., 2015). For example, Gray et al. (2000) analysed the forms of soil Cd in 12 different New Zealand pasture topsoil using a sequential fractionation procedure. They observed that there was a wide range in the concentration of Cd associated with individual soil fractions and large variations between soils. However, on average for all soils, they observed that the smallest proportion of Cd was in the exchangeable form (3%), and the greatest proportion of Cd was associated with the organic fraction (34%). Similarly, Krishnamurti et al. (1996) determined the speciation of soil Cd in two contrasting soil types (typic boroll and udic boroll), under two different wheat cultivars (Arcola and Kyle) in Saskatchewan. They found that Cd

was dominated by association with organic matter in the typic boroll rhizosphere soil under both Arcola (0.12 mg Cd/kg) and Kyle (0.07 mg Cd/kg) cultivars. They suggested that the secretion of LMWOAs into the rhizosphere soil increased the organically bound fraction of Cd in the rhizosphere soil of both plants. In another study, Cd speciation in sandy loam soil collected from vegetable fields decreased in the order residual (44.5%)> acid extractable (27.96%)> reducible (Fe/Mn oxide) (23.09%)> oxidizable (organically bound) (4.5 %) (Wang et al., 2018). Jingchun et al. (2008) reported that the exchangeable Cd fraction in the rhizosphere soil of a mangrove forest soil was greater than the other fractions. However, they reported that the exchangeable Cd percentage in the rhizosphere was significantly lower than the exchangeable Cd percentage in the bulk soil. They attributed this discrepancy to plant uptake, and the complexing and chelating of Cd by soluble exudates of roots. Ru et al. (2006) reported that at soil Cd concentrations of 60 mg Cd/kg, the exchangeable Cd concentration (DTPA-extractable Cd) in the rhizosphere soil (31.45 mg Cd/kg) was significantly ($P<0.05$) lower than that in the non-rhizosphere soil (38.5 mg Cd/kg) for Indian mustard (*Brassica juncea*). These authors suggested that this species may take up more Cd from near the root and that removed Cd cannot be quickly replenished from soil further away from the roots.

2.8 Soil factors affecting Cd bioavailability in soil

Many soil factors are known to influence the bioavailability of Cd and other metals in the soil. Soil pH and soil organic matter (SOM) are the dominating soil factors widely reported to influence Cd bioavailability (Table 2.7) (McLaughlin and Singh, 1999).

2.8.1 Soil pH

The bioavailability of cationic trace metals is negatively correlated with soil pH (Loganathan et al., 2008; Shahid et al., 2016) (Table 2.7). Many studies have indicated that pH is the dominant soil factor that determines Cd bioavailability in soil (Meng et al., 2018). Xian and Shokohifard (1989) observed a decrease in the concentration of Cd associated with soil carbonates (from 1.41 to 0.58 mg Cd/kg) when soil pH was reduced from 7.0 to 4.6, and this increased the concentration of exchangeable soil Cd from 5.9 to 7.4 mg Cd/kg, subsequently increasing the bioavailability of Cd in loam soil. Loganathan (2012) showed that Cd sorption onto metal oxides increased when the soil pH increased from 4 to 8 and attributed this to the 'sorption edge'. Naidu et al. (1994) investigated the effect of pH on the sorption of Cd in four soil types (Xeralf, Andep, Oxisol (Malanda), Oxisol (Mena)) from Australia and New Zealand. They observed greater (approximately 100%) Cd sorption in all four soil types at high soil pH (>7) (Figure 2.5). They explained that at higher pH the stable species of Cd ($CdOH^+$) has increased sorption to soil surfaces.

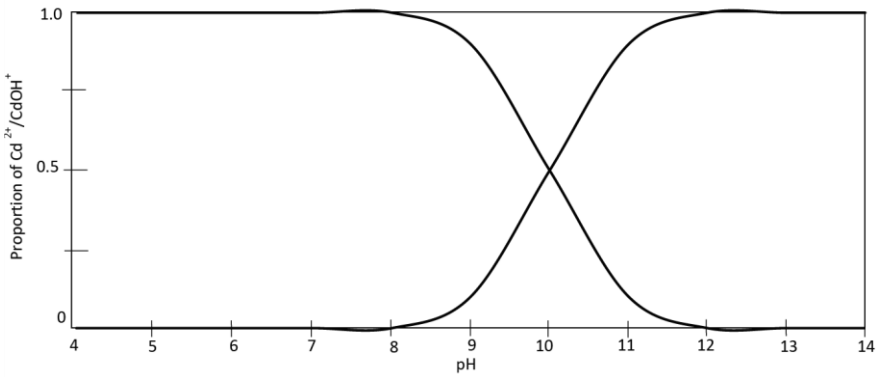

Figure 2.5. Effect of pH on the proportion of Cd^{2+} and $CdOH^+$ species in solution (redrawn and adapted from Naidu et al. (1994)).

Similarly, a study conducted by Tahervand and Jalali (2016) on Cd sorption and desorption in four calcareous soils in Iran reported that the proportion of soil Cd as the free Cd^{2+} species decreased with increasing soil pH from 2 to 8. They also reported that $CdOH^{+}$ was present as the predominant species when pH was greater than 8. Christensen (1984) observed that Cd sorption by sandy and loamy soils increased by a factor of 3 for every pH unit increase between 4 and 7.7. In addition, Appel and Ma (2002) reported that a unit increase in pH of oxisol soil in Puerto Rico resulted in a 36% increase in Cd sorption. A study by Gray et al. (1998) using Te Kowhai silt loam and Waiotira silt loam (pH 4.8-6.2) in New Zealand found that an increase in soil pH from 5.1 to 6.1 resulted in an increase in Cd sorption to the soil by up to 48% and 26%, respectively. However, Naidu and Harter (1998) showed that there was no significant change in exchangeable Cd concentration when pH was increased above 5.5. They also reported that there was a significant increase in the concentration of organic matter extractable Cd which suggests that more Cd is sorbed into the soil by complexation with organic ligands at high pH. They suggested that above a soil pH of 5.5, competition for complexation between the metal ion and organic ligand anion is greater than the affinity of the metal ion on the soil surface. Meng et al. (2018) conducted a field experiment to evaluate the effects of alkaline amendments on soil bioavailability of Cd in rice grown in a Cd-contaminated clay loam soil. They suggested that an increase in soil pH can directly transform the bioavailable Cd fraction to a more stable fraction by Cd immobilisation in soils.

Table 2.7. Major factors affecting the Cd bioavailability in soil.

Factor	Cd bioavailability in soil	Reference
pH	Negative correlation between soil pH and Cd bioavailability in soil. Low soil pH (<6), Cd in soil solution is predominately present in the free Cd^{2+} form as greater H^+ concentration competes with Cd^{2+} for sorption sites. High soil pH (>6) neutralises H^+ ions on the surface -OH groups associated with organic matter and clay minerals, creating a larger specific-sorption sink for Cd.	Williams and David (1973) Tsadilas (2000) Xian and Shokohifard (1989) Loganathan et al. (2008) Shahid et al. (2016)
Soil Organic Matter (SOM)	Humic substances, the largest constituent of soil organic matter, contributes to the majority of cation binding properties. Ionic interactions exist between Cd^{2+} and the overall net negative charge of humus, reducing Cd bioavailability. Surface OH groups associated with phenolic groups in humus provide strong specific sorption by forming multidentate bonds with Cd^{2+}.	Gray et al. (1998) Loganathan et al. (2008) Shahid et al. (2016)
Clay mineralogy, Fe and Mn oxide/ hydrous oxide	The affinity between Cd and O is strong to remove H^+ ions from metal -OH groups, resulting in a strong covalent bond between Cd^{2+} and the exposed metal oxide.	Backes et al. (1995) Essington (2004)

2.8.2 Soil organic matter

The amount and characteristics of SOM is also a critical factor influencing Cd bioavailability (Loganathan, 2012); negatively charged functional groups in SOM (e.g. carboxylic and phenolic hydroxyl groups) may form complexes with Cd and reduce Cd bioavailability (Zaho et al., 2014) (Table 2.7). Gray et al. (1998) reported that the soil organic C percentage in different types of soil collected from agricultural lands in New Zealand decreased in the order Allophanic soil (te kuiti silt loam-11.5%)> Melanic soil (waiareka clay loam-6.9%)> Gley soil (temuka clay loam-5.9%)> Pallic soil (takahe silt loam-2.6%), where the highest total Cd concentration was observed in Allophanic soil (0.48 mg Cd/kg) followed by Melanic soil (0.35 mg Cd/kg), Gley soil (0.12 mg Cd/kg) and Pallic soil (0.10 mg Cd/kg). Zanders et al. (1999) reported that the total Cd

concentration decreased from 0.64 to 0.12 mg Cd/kg when soil organic carbon decreased from 16.8 to 6.7% across a soil depth from 0-300 mm in a well fertilised, hill-country Allophanic soil under long-term pastoral land use in New Zealand. Zhao et al. (2014) reported that the chemical removal of soil organic matter from acidic purple paddy soil (APPS) (13.92 g/kg soil organic matter) and calcareous purple paddy soil (CPPS) (29.87 g/kg soil organic matter) significantly decreased the amount of Cd^{2+} adsorbed on APPS and CPPS by 4.32-15.78% and 9.05-19.02%, respectively. They suggested that the organic matter was beneficial to the immobilisation of Cd in soils through the adsorption process; however, its contribution varied depending on soil type as the Cd desorption percentage was much greater on CPPS than on APPS. Yu et al. (2016) suggested that the bioavailability of trace metals could be decreased through the adsorption or the formation of stable complexes with functional groups in SOM. However, the authors did not observe a correlation between SOM and metal availability due to low SOM content (varied only from 0.92% to 3.9%) of the soils used in this study. Recently Stafford (2017) used Allophanic and Gley soil to examine the relationship between soil C and total soil Cd concentration. They reported that total soil C was significantly ($P<0.001$) correlated ($R^2=0.87$ (Allophanic) and $R^2=0.83$ (Gley)) with soil total Cd concentration and accounted for a large proportion (83-90%) of the variability in soil total Cd concentrations.

2.9 Influence of rhizosphere functions on Cd availability and plant Cd uptake

The rhizosphere is the volume of soil around living plant roots that is influenced by root activity (Hinsinger, 1998; Lux et al., 2010). Generally, Cd bioavailability depends on the physical, chemical and biological processes operating in the rhizosphere (McLaughlin

and Singh, 1999). These processes are related to many factors such as; changes in rhizosphere pH, the activity of plant root-associated microorganisms, and the secretion of organic compounds from plant roots to the rhizosphere such as siderophores, high molecular weight compounds, and low molecular weight organic acids (Jeyakumar et al., 2010; Lux et al., 2010).

2.9.1 Rhizosphere pH

The rhizosphere soil solution pH differs by up to 2.5 pH-units from that of the bulk soil solution, depending on the plant species and the buffering capacity of the bulk soil (Youssef and Chino, 1989). This difference can be attributed to a combination of mechanisms, including (i) cation exchange capacity (ii) root exudation and respiration (iii) redox-coupled processes involving changes in the oxidation state of Fe and Mn (Hinsinger et al., 2006). Shuman and Wang (1997) conducted a study on the effect of soil pH on Cd bioavailability in the rhizosphere and bulk soil under paddy rice (*Oryza sativa*). They found that the mean rhizosphere soil pH (5.11) was less than the bulk soil pH (5.20) and the mean exchangeable Cd concentration of the rhizosphere soil (2.5 mg Cd/kg) was higher than the bulk soil (2.0 mg Cd/kg). Krishnamurti et al. (1996) also observed lower rhizosphere pH (7.75) than bulk pH (7.95) in a typic boroll soil under wheat cultivars and found the corresponding Cd concentration was higher in rhizosphere soil than bulk soil. Jingchun et al. (2008) observed a significantly reduced pH (5.94) in the rhizosphere for mangrove plants grown in sediment soil with a Cd concentration of 10 mg Cd/kg relative to bulk soil (pH 6.40). However, they found that the percentage of the Cd concentration in the exchangeable fraction of the rhizosphere soil was 20% while that for bulk soil was a 25% percentage. They suggested that this discrepancy may be due to the increased plant

Cd uptake from the rhizosphere soil compared to the bulk soil (Ru et al., 2006; Jingchun et al., 2008).

2.9.2 Rhizosphere Microorganisms

Rhizosphere microorganisms are symbiotically associated with the roots of most plant species. Microorganisms obtain food from the rhizosphere, and in return, they regulate the uptake of plant nutrients and trace elements (Jones, 1998). Rhizosphere microorganisms can have either a positive or negative correlation with plant Cd bioavailability in soil depending on microorganism and plant species interactions. The uptake of Cd by plants is associated with the presence of microbially-derived siderophores and ligands which influence the chemistry of Cd in the rhizosphere soil (Jones, 1998); such molecules may reduce soil pH facilitating an enhancement of Cd bioavailability (Wang et al., 2009). A study conducted by Chauhan and Rai (2009) found a 2.5 and 1.8-fold increase in Cd uptake by Indian mustard plants in the presence of *Pseudomonas fluorescens* and *Trichoderma harzianum*, respectively. Guo et al. (1996) reported that soil micohorrizal fungus (*Glomus mosseae*) increased the uptake of Cd by up to 37% and 41% in beans (*Phaseolus vulgaris*) and maize, respectively. Chen et al. (2017) reported that the endophytic bacterium *Pseudomonas fluorescens* significantly increased the Cd accumulation in *S.alfredii* root and shoot by 46% and 60%, respectively at 1.2 g/L Cd concentration in hydroponic solution. These studies provide strong evidence that the secretion of exudates to the soil solution by microorganisms increases Cd bioavailability (Li et al., 2007; Chauhan and Rai, 2009).

There is, however, contrasting evidence of the microbiological effect on Cd uptake. Dary et al. (2010) reported *Bradyrhizobium sp. 750 + Ochrobactrum sp. Azn6.2 + Pseudomonas* in soil decreased the uptake of Cd in yellow lupin (*Lupinus*) (from a plant

concentration of 4.00 to 0.25 mg Cd/kg). They concluded that these bacterial species secrete mammalian metallothioneins, which Cd can bind to the cell surface and reduce Cd uptake by plants. Similarly, Tripathi et al. (2005) reported that *Pseudomonas putida* decreased the Cd concentration in roots of mung bean plants (*Vigna radiata*) from 24 to 12 mg Cd/kg. They observed that these bacteria secrete the compound pyoverdine, which binds with Cd to reduce Cd bioavailability.

2.9.3 Presence of competing metal ions in the soil

Cadmium uptake can be influenced by interactions with other cations such as Ca^{2+}, Fe^{2+}, Zn^{2+}, Se^{2+} in the rhizosphere soil solution (Lux et al., 2010). These cations can cause an impact on Cd uptake due to the competition for cation transport-specific channels or carriers with Cd (Shahid et al., 2016). Li et al. (2012) showed that application of the Ca^{2+} channel blocker 'verapamil', significantly reduced Cd^{2+} influx into seepweed (*Suaeda*) roots by 52%, in an agar medium. In another example, Hu et al. (2014) reported a decrease in rice grain Cd concentration by 44% through increasing the Se concentration in paddy soil from 0-5 mg Se/kg in soil.

Cadmium and Zn have similar chemical characteristics and behave alike in the soil- water- plant system (Mengel et al., 2001). The similarities of chemical behaviour between these two metals result in a protective effect of Zn against Cd uptake. As a consequence, plants absorb more Cd when the soil solution is Zn deficient (Mengel et al., 2001). For example, Zhao et al. (2006), in the analysis of Cd and Zn interactions in rockcress (*Arabidopsis Halleri*), found that increasing the amount of Zn in a hydroponic culture from 0 to 65 mg Zn/L resulted in decreased Cd accumulation by 38% and 81%, in the root and shoot, respectively. Similarly, Han et al. (2006) reported that Cd accumulation in maize roots increased 3-fold under Zn deficiency in hydroponic culture,

whereas there was an 87% reduction in total root Cd when the Zn concentration increased from 0 to 0.16 mg Zn/L.

Iron is another antagonistic micronutrient that competes with Cd for uptake in plants when they co-occur in soil solutions (McLaughlin and Singh, 1999). Ueno et al. (2008) reported that the introduction of 2.8 mg Fe/L to hydroponic culture reduced the Cd concentration in rockcress by 3.6-fold.

2.9.4 Rhizosphere root exudates

Root secreted Cd complexing ligands (root exudates) can be categorised into either; organic or inorganic ligands (Bali et al., 2020) (Figure 2.6). Organic ligands from root exudates can be categorized as either high molecular weight or low molecular weight materials (Luo et al., 2014). The Low Molecular Weight Organic Acids (hereafter Low Molecular Weight Organic Acids described as LMWOAs) such as malic, oxalic, acetic, fumaric and citric acids are negatively charged anions that are capable of forming stable complexes with bioavailable Cd^{2+} in soil and influence Cd uptake by plants (Han et al., 2006; Zhu et al., 2011). The composition of organic acids released from roots is highly variable and depends on plant species and cultivars (Bao et al., 2011).

A study conducted by Javed et al. (2017) reported that enhancement of oxalic and acetic acid secretion by 111% and 631%, respectively, increased the root Cd concentration from 0.01 to 0.07 mg Cd/kg, while shoot Cd concentration increased from 0.01 to 0.03 mg Cd/kg in maize (3062 cultivar) (Table 2.8). Fu et al. (2018) reported that high Cd-accumulating rice plants exude more total LMWOAs than low-Cd accumulating rice plants and that this influences plant Cd uptake (Table 2.8). They observed that the total

LMWOA concentration secreted by a high Cd uptake rice cultivar was 136% higher than for a low-Cd accumulating cultivar at a 5 mg Cd/L treatment in a hydroponic experiment.

In contrast, Zhu et al. (2011) found 3.5 times higher root secretion of oxalic acid in a low-Cd accumulating tomato cultivar than a high-Cd accumulating tomato (*Solanum lycopersicum*) cultivar. The low-Cd accumulator had a 75% ($P<0.05$) reduced shoot Cd concentration for a 1 mg Cd/L hydroponic treatment. They suggested that root-secreted oxalate for the low Cd accumulating tomato cultivar plays an important role in reducing Cd toxicity in tomato by excluding the entry of Cd into the root cell membrane. Furthermore, Oloumi et al. (2011) reported that the addition of fumaric acid (5 mg/L) to the growth media significantly ($P<0.05$) reduced the total Cd concentration in canola seedlings (*Brassica napus*) by 98% compared to the control treatment in the presence of 1 mg Cd/L in the growing media.

Table 2.8. Findings of previous studies on the effect of LMWOAs on metal uptake by different plant species.

Plant species	Concentration of the metal	LMWOAs	Variation in tissue Cd concentration	Effect of LMWOAs on metal uptake	References
Rice **Lu527-8 high Cd accumulating cultivar** **Lu527-4 - low Cd accumulating cultivar**	0-5 mg Cd/L	Oxalic Malic Acetic	Shoot and root Cd concentration in Lu527-8 increased by 320% and 270%, respectively with increase of Cd from 0 to 5 mg Cd/L. Shoot and root Cd concentration in Lu527-8 increased by 110% and 90%, respectively with increase of Cd from 0 to 5 mg Cd/L.	High Cd accumulated variety secreted more LMWOAs. LMWOAs bind with Cd^{2+} ions due to the carboxyl functionality and reduce Cd^{2+} ion toxicity. The Cd-LMWOAs complexes will reach to vascular region of the root or they can chelate around root apex to reduce Cd toxicity inside plant.	Fu et al. (2018)
Maize (3062 v)	0-5.62 mg Cd/kg	Oxalic Acetic	Shoot Cd concentration increased by 600% with increase of Cd from 0 to 5.62 mg/L. Root Cd concentration increased by 200% with increase of Cd from 0 to 5.62 mg Cd/L.	Oxalic acid has more negative charges than acetic acid to bind with Cd^{2+} ions. Cd-LMWOAs complexes alleviate the toxicity of free Cd^{2+} ions inside the plant mainly accumulating in roots.	Javed et al. (2017)
Black nightshade (Solanum nigrum- high Cd accumulating plant species)	1-20 mg Cd/kg	Oxalic Citric	Shoot and root Cd concentration in Black nightshade increased by 420% and 566%, respectively with increase of Cd from 1 to 20 mg Cd/kg.	LMWOAs influence the rate of Cd desorption (release) from different soil. Cd-LMWOAs complexes enter plant by penetrating through lipid layer of root membrane.	Bao et al. (2011)
Hot pepper Cultivars (Capsicum frutescens) **JFZ (high Cd accumulating cultivar)** **YCT (low Cd accumulating cultivar)**	0-1.2 mg Cd/L	Oxalic Tartaric Acetic	Shoot Cd concentration in JFZ increased by 203% with increase of Cd from 0 to 1.2 mg Cd/L. Shoot Cd concentration in YCT increased by 196% with increase of Cd from 0 to 1.2 mg Cd/L.	JFZ secrets more Oxalic and tartaric acids. Cd-Oxalic/Cd tartaric complexes penetrate via root membrane to increase the Cd uptake. YCT secrets more acetic acid and Cd-acetic complex immobilise in the root.	Xin et al. (2015)
Ramie (Boehmeria nivea)	0-10 mg Cd/L	Citric	Shoot Cd concentration increased by 103% with increase of citric acid concentration from 0 to 283.5 mg/L. Root Cd concentration increased by 26%, with increase of Cd with increase of citric acid concentration from 0 to 283.5 mg/L.	Cd-citric acid complex enter plant tissues through breaking in the root endodermis and Casparian strips.	Li et al. (2014)
Sedum alfredii	0-1.1 mg Cd/L	Oxalic Succinic Fumaric	-	Oxalic acid can enhance the adsorption and desorption of Cd in soil and form a Cd-oxalate complex via an oxalate bridge between the surface and the Cd^{2+} ion. LMWOAs-Cd complexes can enhance the Cd uptake by plant.	Luo et al. (2015)
Rapeseed (Brassica napus)	1.1 mg Cd/L	Citric	Shoot Cd concentration increased by 33% with increase of citric acid concentration from 0 to 1.8 mg/L. Root Cd concentration increased by 44%, with increase of citric acid concentration from 0 to 1.8 mg/L.	Organometallic complex formation in the soil solution can act as carriers for Cd^{2+} ions towards root surface. These complexes can disconnect into the free Cd^{2+} at the root surface which can and immersed by root membrane.	Ehsan et al. (2014)

2.10 Plant Cd uptake, distribution and translocation

2.10.1 Cadmium complex with organic acids in root exudates

The secretion of anionic LMWOAs to the rhizosphere soil leads to a cation-anion imbalance inside the plant cytosol. To balance this, plants efflux H^+ ions from plant cells (plant cytosol) into the rhizosphere soil from the proton pump (Luo et al., 2015; Tanwir et al., 2015) and any decrease in the rhizosphere pH due to this function will increase Cd bioavailability which in turn influences Cd uptake by plants. Studies have suggested that Cd chelation with LMWOAs may create an important pathway to mitigate the toxicity of free reactive metal ions inside the plant (Pence et al., 2000; Wei et al., 2007). Researchers have reported several mechanisms which describe how Cd-LMWOA complexes enhance Cd uptake by plants, and these are described below:

(i) Cadmium can complex with organic acids to produce mobile and soluble organically bound Cd complexes, which can penetrate the lipid membrane of root cells. For example, Xin et al. (2015) reported that high Cd accumulating hot pepper roots (cultivar JFZ) secrete elevated levels of oxalic and tartaric acids to the rhizosphere soil. Both oxalic and tartaric acid have di- and tri- carboxylic acid functionality and high formation constants with Cd (Oxalic pKa-3.71, tartaric pKa-2.98) which can form complexes with free Cd^{2+} ions in the soil solution and increase Cd uptake via root membranes (Table 2.8). Krishnamurti et al. (1997) reported that the addition of citric (1.92 mg/kg) and oxalic acid (0.90 mg/kg) significantly increased Cd bioavailability in soil by 125% and 75%, respectively within 8 hrs. They explained that increased Cd bioavailability in the presence of LMWOAs is mainly due to the formation of Cd-organic acid complexes in the soil.

(ii) Cadmium-organic acid complexes might enter plants by breaking the endodermis and Casparian strips of the root cells (Li et al.,2014) (Table 2.8).

(iii) Organometallic complexes in the soil solution can act as a carrier for Cd^{2+} ions towards the root surface and these complexes can disassociate into free Cd^{2+} at the root surface which can be absorbed by root membranes. For example, Han et al. (2006) suggested that acetic acid, which has a lower formation constant with Cd (pKa=1.50), can act as a carrier for Cd^{2+} ions towards maize roots and lead to the disassociation of Cd-acetic complexes into free Cd^{2+} ions at the root surface which can be absorbed by the maize root membrane (Table 2.8).

In contrast, the secretion of LMWOAs into the rhizosphere can also contribute to *ex planta* Cd detoxification mechanisms by inducing the formation of metal-organic acid complexes in the rhizosphere soil which immobilize contaminants before they enter the root membrane. For example, Oloumi et al. (2011) reported that the formation of Cd-fumaric complexes reduces Cd bioavailability in nutrient solution and thereby reduces Cd uptake by canola seedlings due to the formation of immobile ternary surface complexes in the media. Similarly, Kazemi Movahed (2020) reported that secretion of fumaric acid reduced the solubility and bioavailability of Cd for uptake by the soybean plant (*Glycine max*), through the formation of Cd-organic acid complexes in the soil. These authors suggested that steric factors associated with complexes that are too large to cross root membranes easily prevent Cd influx into the root cells.

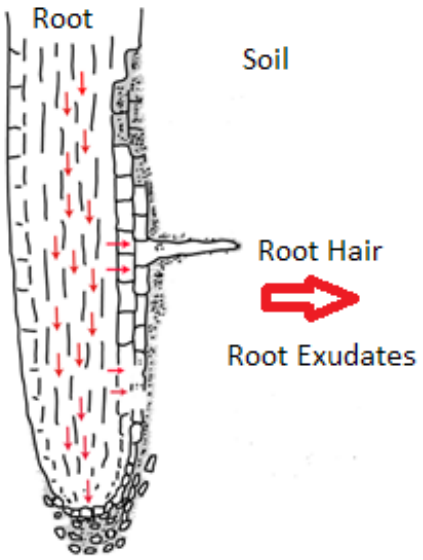

Figure 2.6. Pathway of root exudates inside the root (redrawn and adapted from Akter (2016)).

2.10.2 Cd transport through the root membrane

The root epidermal layer in plants acts as a barrier for diffusing Cd from soil to plant. Cadmium ions (Cd^{2+}) combine with specific and non-specific transporters such as ZIP, OsIRT, Yellow-Stripe 1-Like protein, and Low-affinity Cation Transporter (LCT1) of essential elements (Fe^{2+}, Ca^{2+} and Zn^{2+}) (Llugany et al., 2012). In doing so, Cd can pass through the corresponding ion channels of these molecules and subsequently enter through the root epidermis layer (Roth et al., 2006; Mendoza-Cózatl et al., 2011). Various transcriptional and post-transcriptional genes are responsible for encoding specific and non-specific transporters for the uptake of essential elements such as Zn^{2+} and Fe^{2+} (Roth et al., 2006). It was reported that the ZIP family transporters which are responsible for Fe^{2+} and Zn^{2+} uptake also mediate Cd uptake by plant roots (Mendoza-Cózatl et al., 2011). Han et al. (2006) reported that the Zn membrane transporter ZIP enhanced Cd uptake 3-fold in maize when there is a Zn deficiency in the soil. The OsIRTs transporter, which

is considered as a transporter for Fe^{2+} uptake, is also suggested to contribute to Cd uptake by plants (Nakanishi et al., 2006; Lee and An, 2009). For example, Lombi et al. (2002) reported that OsIRT enhanced Cd uptake in alpine pennycress (*Thlaspi caerulescens*) by 3-fold in a Fe deficient hydroponic culture relative to a Fe sufficient hydroponic culture. Cohen et al. (1998) reported that Fe deficiency in pea (*Pisum sativum*) induced the expression of Fe transmembrane carriers (IRT1) and found that Cd uptake was 7-fold higher when the hydroponic culture had no Fe in comparison to a solution with a Fe value of 0.5 mg Fe/L.

2.10.3 Subcellular distribution of Cd in plant root

Despite no known essential role for plant growth, Cd can be absorbed by plant roots and accumulated in different plant tissues (Senden and Wolterbeek, 1990) (Figure 2.7). Subcellular accumulation of Cd in plants varies greatly depending on plant species and cultivar (Huang et al., 2019). Studies have reported that for most plants, Cd tends to accumulate in the roots, with only a small portion being translocated to aerial parts where roots act as an effective barrier to Cd translocation to shoots (Li et al., 2019a).

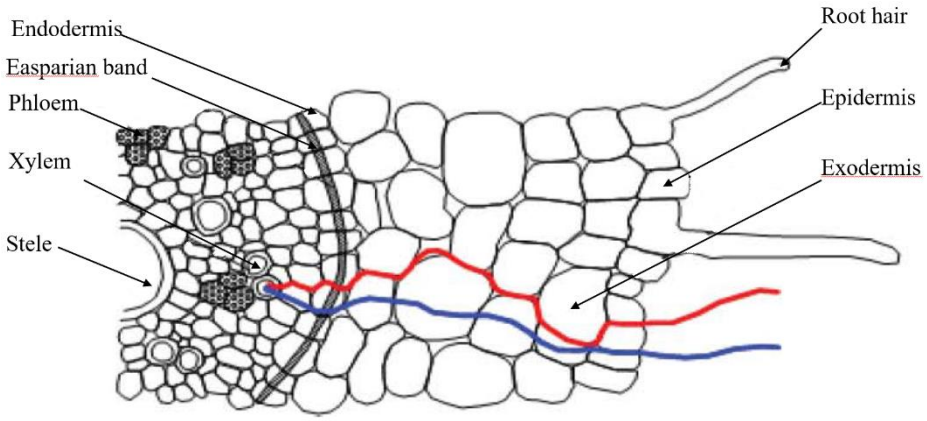

Figure 2.7. Movement of Cd plants apoplastic and symplastic pathways of root (redrawn and adapted from Song et al. (2017)).

Movement of Cd through root cells can either be passive via diffusion and convection through the apoplast (apoplastic pathway) or active and selective transport from cell to cell via the symplast (symplastic pathway) (Figure 2.7) towards xylem vessels (Song et al., 2017). However, when Cd^{2+} is moving via the apoplast pathway of root cells it is adsorbed into negatively charged sites present in the root cell wall (Senden and Wolterbeek, 1990; Conn and Gilliham, 2010). Table 2.9 summarises studies that have been conducted to determine the subcellular distribution of Cd in different plant species. Zhou et al. (2015) reported that 60-90% of absorbed Cd was accumulated in the roots of 32 hybrid rice cultivars (Table 2.9). Lozano-Rodriguez et al. (1997) observed that Cd accumulation in plant roots varies with plant type and they observed that more Cd accumulates in the cell-wall fraction of roots of corn (40%) than pea (20%). Wu et al. (2005) showed that 51% of Cd existed as a soluble form and 36% was bound in the cell walls of barley roots. Yu et al. (2021) reported that Cd was mainly distributed in the soluble fraction (vacuoles) (47%-48%) and cell wall fraction (42%-45%) in the roots of rice (Lu527-8). Similarly, Isaure et al. (2006) observed that Cd was mainly (75%) bound to O/N groups in the root cell wall of thale cress *(Arabidopsis thaliana)* with a small proportion (about 25%) of Cd bound to S-containing ligands in the root cell wall. Li et al. (2019a) suggested that Cd in the soluble fraction of water thyme (*Hydrilla verticillate*) was mainly accumulated in cell vacuoles which is a dynamic organelle that occupies as much as 90% of the total cell volume in some cell types. Furthermore, they reported that vacuoles contain various organo-ligands, such as sulfur-rich peptides and organic acids to bind with free Cd^{2+} ions. In sweet potato (*Ipomoea batatas*) plants, the cell wall has many negatively charged sites on the surface of cellulose, hemicellulose, pectin, and protein components, which can bind Cd ions and restrict their transport across the membrane (Huang et al., 2019) (Table 2.9).

Table 2.9.Previous studies conducted on the subcellular distribution of Cd in different plant species.

Details	Findings	Reference
Maize and pea plants were grown in nutrient solution with 1 and 5 mgCd/L concentrations for 11 days.	• Both plants showed high Cd accumulation in roots compared to shoots. • The root Cd accumulation increased by 49%, and 84% in maize and pea, respectively when the nutrient Cd concentration increased from 1 to 5 mg Cd/L. • More root Cd accumulates in the cell-wall fraction of roots of corn (40%) than pea (20%).	Lozano-Rodriguez et al. (1997)
A hydroponic experiment conducted to asses the subcellular distribution of Cd in barely roots under 0.05 µg Cd/L and 5.00 µg Cd/L Cd treatments.	• Mainly Cd existed as a soluble form (51%) in barley roots • There was 36% of Cd were bound in cell walls in barley roots and 3% were bound to chloroplast /trophoplast.	Wu et al. (2005)
The subcellular distribution of Cd in plant tissue investigated in (*raddish Raphanus sativus*) (Cd sensitive cultivar) under different Cd concentrations in soil; 0,1.0, and 5 mg Cd/kg	• Cd accumulation in roots of Cd sensitive cultivar was mainly associated with cell walls (46-49%) and followed by the soluble fraction (36–38%) organelles (15%).	Xin et al. (2017b)
A hydroponic experiment conducted to understand the characteristic mechanism of high Cd accumulation in rice roots (variety Lu527-8) under increasing Cd concentration from 0 to 5 mg Cd/L.	• Cd was mainly bound in the soluble fraction (47%-48%) and cell wall fraction (42%-45%).	Yu et al. (2021)
A field experiment conducted to determine the variations of plant tissue Cd accumulation in 32 hybrid rice cultivars in China.	• Cd mainly accumulates in rice roots (60%).	Zhou et al. (2015)

2.10.4 Xylem loading of Cd

Xylem loading is the process where plant absorbed Cd is diffused to the stem xylem from root cells (Figure 2.8) (Clemens et al., 2002). This plays an important role in Cd accumulation within aerial plant parts (Mori et al., 2009). Many studies have identified a number of important membrane transporters such as ATP Binding Cassette (ABC) superfamily, HMA (Heavy Metal ATPase), ZIP (ZRT, IRT-like protein) and YSL (Yellow-Stripe-Like Transporter) which may be involved in Cd loading into the xylem

sap for internal plant transport (Hasanuzzaman et al., 2018; Shahid et al., 2016; Song et al., 2017).

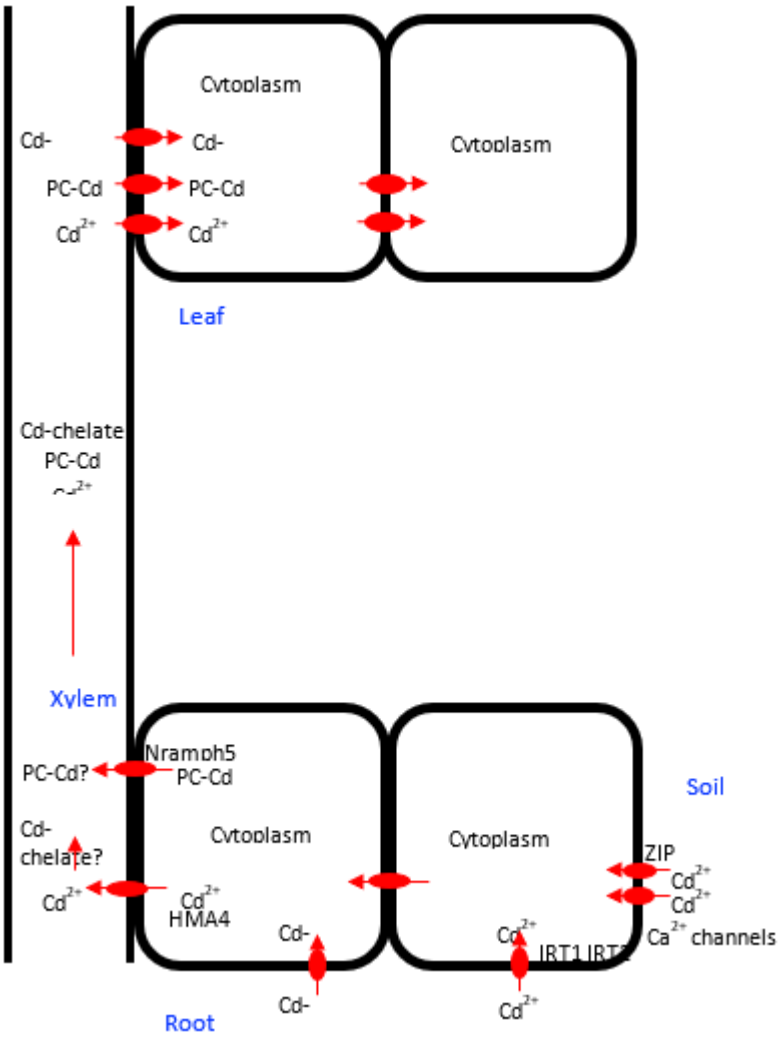

Figure 2.8. Cd translocation pathway through plant species (redrawn and adapted from (Song et al. (2017)).

Several studies have found that P1B-ATPase and ZIP family transporters are also involved in Cd transfer across the plasma membrane into shoots (Hanikenne et al., 2008; Wong and Cobbett, 2009). Over-expression of OsHMA3 has been found to improve Cd tolerance and reduce accumulation in rice. Sasaki et al. (2014) reported that over-expression of OsHMA3 improved plant tolerance to Cd in brown rice and decreased Cd accumulation by about 81% compared with wild-type plants. However, stem xylem

loading is a tightly regulated process mediated through mostly unidentified membrane transport proteins which are specific for plant species and cultivars.

2.10.5 Cadmium translocation through xylem

Cadmium can translocate from roots to other tissues via transpiration-driven xylem loading, via symplast and apoplast pathways (Figure 2.8) (Shahid et al., 2016). Many studies have highlighted the influence of type and concentration of LMWOAs in xylem sap on Cd translocation in different plants (Table 2.10).

Cadmium mainly binds with negatively charged sites present in the xylem cell wall during the translocation of Cd^{2+} to shoots via the xylem vessels (Senden and Wolterbeek, 1990). Xylem sap contains amino acids, proteins and LMWOA such as citric, maleic, oxalic which can form complexes with Cd^{2+} (Ueno et al., 2005). The formation of Cd complexes in xylem sap prevents Cd^{2+} adsorption to cell walls and hence, facilitates the movement of Cd complexes with xylem sap to upper plant tissues (Álvarez-Fernández et al., 2014) (Figure 2.8). These LMWOA-Cd complexes may be involved in the metal detoxification process leading to a reduction of the free ionic forms of metal inside the plant (Pence et al., 2000). Senden et al. (1995) reported that citric acid was the major ligand for Cd transport in tomato plants, where >50% of Cd is transported as a Cd-citric acid complex. Li et al. (2019b) reported that citric (R=0.90), tartaric (R=0.73) and oxalic acid (R=0.90) concentrations in the xylem sap of the Luhui17 rice cultivar were significantly and positively correlated with xylem sap Cd concentration. Fu et al. (2019) found that the concentration of citric and tartaric acids in rice (Lu.527-8) xylem sap was positively correlated with the total xylem sap Cd concentration when the soil Cd concentration increased from control (0.31 mg Cd/kg) to 5 mg Cd/kg soil (R=0.82 and R=0.97, respectively) (Table 2.10).

Table 2.10. Summary of previous studies conducted on the effect of trace metals on plant xylem sap LMWOAs concentration.

Plant specie	Metal concentration in the media	Type of LMWOA produced in the xylem	Summary of the study	References
Rice •LU527-8 (high Cd accumulating Cultivar) •LU527-4 (low Cd accumulating cultivar)	Cd (0-10 mg Cd/kg)	Malic Tartaric Citric oxalic	• LU527-8 has high LMWOAs concentration compared to LU527-4 xylem sap at different Cd concentrations ranged from (0-10 mg Cd/kg). • The citric acid (R=0.82) and tartaric (R=0.97) acid concentration significantly and positively correlated with xylem sap Cd concentration of LU527-8.	Fu et al. (2019)
Rice •A low Cd-accumulating rice line D62B •A common rice line Luhui17	Cd (0-2.0 mg Cd/L)	Malic Citric Tartaric Oxalic	• The tartaric (R=0.88), malic (R=0.77) and citric acid (R=0.89) concentrations were significantly and positively correlated with Cd concentration in xylem sap of D62B. • Citric (R=0.90), tartaric (R=0.73) and oxalic acid (R=0.90) concentrations significantly and positively correlated with the xylem sap Cd concentration of Luhui17. • Citric acid formed more stable octahedral complexes with metal cations in xylem sap and increased the Cd translocation.	Li et al. (2019b)
Castor beans	Cd (0-1mg/L)	Citric	• Less than 10% of Cd bind with citric and glutathione and translocate in the xylem. • More than 90% translocate as free Cd^{2+} ions in xylem sap.	Hazama et al. (2015)
Rock cress	Cd (3.9 mg/L)	Malic Citric	• Malic or citric did not participate in Cd translocation in xylem sap while Cd mainly translocated as free Cd^{2+} ions (86%) in the xylem sap.	Ueno et al. (2008)

However, Hazama et al. (2015) observed that Cd in castor beans (*Ricinus communis*) is mainly translocated as free Cd^{2+} ions (>90%) rather than as a complex with organic acids. Similarly, Ueno et al. (2008) reported that there was a higher percentage (85.7%) of free Cd^{2+} concentration in the xylem sap of rockcress compared to citric (3.2%) and malic (0.4%) acids when exposed to a Cd solution concentration of 1 mg Cd/L. They concluded that Cd translocation in rockcress is an energy-dependent process and that free Cd^{2+} does not need to be complexed to organic acids for translocation.

2.11 Analytical methods to measure LMWOAs and Cd species in plant saps

A number of advanced analytical techniques can be used to quantify the form and amount of LMWOA and Cd species in plant saps, including High-Performance Liquid Chromatography (HPLC) and ^{113}Cd-Nuclear Magnetic Resonance Spectroscopy (^{113}Cd-NMR). Table 2.11 shows the various methodologies that have been used to measure the form of Cd in different xylem saps.

2.11.1 High-Performance Liquid Chromatography (HPLC)

High-Performance Liquid Chromatography (HPLC) can be used to identify organic compounds in xylem saps (Fu et al., 2019) and root exudates (Cawthray, 2003). HPLC separates organic compounds using a reverse-phase column according to their polarity (Arnetoli et al., 2008). In the reverse phase column, organic compounds are partitioned leading to differential migration through the column. As a result, organic compounds elute from the column at different times enabling them to be identified depending on the retention time (Collins, 2004). However, this method has a major limitation for Cd

analysis as it is unable to differentiate between Cd bound to organic complexes and Cd as free Cd^{2+}. This is because organic complexes tend to denature during the analysis (Strobel, 2001). Some studies have used 'Size Exclusion Chromatography (SEC)' to overcome this limitation through modification of the HPLC technique to separate molecules by size or based on hydrodynamic volume (Wei et al., 2007; Kato et al., 2010). Wei et al. (2007) used SEC to analyse the form of Cd in long-distance transport in xylem sap of Indian mustard and they observed that 35% of Cd is transported as organic acid-Cd complexes, and 1% is transported as a Phytochelain-Cd complex.

2.11.2 ^{113}Cd-Nuclear Magnetic Resonance Spectroscopy (^{113}Cd-NMR)

The form of Cd in plant saps can be identified using ^{113}Cd-Nuclear Magnetic Resonance Spectroscopy (Cd-NMR) (Grassi and Mingazzini, 2001). This analysis is carried out by combining the ligand with a stable isotope of Cd (^{113}Cd). When the metal is coordinated with various chelating ligands, the chemical shift of ^{113}Cd differs according to the polarity of the complexed ligand (Larive et al., 1996). Therefore, the ^{113}Cd-NMR technique can differentiate free ionic Cd from complexed Cd in plant saps based on their chemical shifts (ppm). Ueno et al. (2008) reported that Cd is transported as free Cd^{2+} in rockcress xylem sap and they observed a ^{113}Cd-NMR peak corresponding to Cd^{2+} at a chemical shift of 0.27 ppm (Table 2.11). Similarly, Ueno et al. (2005) found that Cd complexed with malate in the leaf sap of alpine pennycress was defined by a ^{113}Cd-NMR peak at a chemical shift of 16.9 ppm. This method has been widely recognised for its high performance in selectivity, reproducibility and sample recovery. However, low sensitivity and high maintenance costs have been noted as key limitations (Emwas, 2015).

Table 2.11. Summary of various methodologies used to measure forms and amount of Cd in different plant species.

Plant species	Methodology	Summary of findings	References
rock cress	Xylem sap was collected from plants exposed to 3.9 mg Cd/L for 9.5 hours. Cd concentration in xylem sap was analysed using Graphite Furnace Atomic Absorption Spectroscopy. The form of Cd in the xylem sap was identified by using the **^{113}Cd-NMR technique**.	• Cd occurred mainly in the free ionic form (85 %) in the xylem sap and the concentration of Cd in the xylem sap increased linearly with increasing Cd concentration in the external solution from 0.05-1.12 mg Cd/kg.	Ueno et al. (2008)
oil seed rape	Xylem sap was collected after plants were exposed to Cd treatment solutions of 2.11 mg Cd/L and 10.57 mg Cd/L for 10 hours. **Size exclusion and high-performance liquid chromatography** was used to investigate the Cd associated chelates in the xylem sap.	• Cd translocated as Cd maleic complex in xylem sap. • Cd concentration in xylem sap linearly increased with xylem sap maleic concentration.	Nakamura and Akiyama (2008)
alpine pennycress	Plants were grown hydroponically in a highly enriched ^{113}Cd stable isotope (10.57 mg Cd/L). **^{113}Cd-NMR spectroscopy** combined with a stable isotope (^{113}Cd) labelling technique was used to identify the form of Cd in the leaves of alpine pennycress.	• Cd was coordinated mainly with malate-83% in the leaves of alpine pennycress. • Cd-NMR with leaf sap showed a signal at the chemical shift of around 16.9 ppm.	Ueno et al. (2005)
rock cress	Leaf sap was collected after the plants were exposed to Cd treatment solutions of 5.28 mg Cd/L and 21.14 mg Cd/L for 9 weeks. **X-ray Absorption Spectroscopy** was used to investigate the Cd associated chelates in the xylem sap.	• Cd (80 %) was coordinated with O atom containing ligand and 20% of Cd coordinated with S atom containing ligand in leaf sap.	Huguet et al. (2012)

2.11.3 Cadmium ion analysis using electrochemical methods: Development of chemically modified carbon paste electrode

Electrochemical methods have become promising tools for Cd analysis due to their speed, low-cost, simplicity and high sensitivity for small size samples. Stripping voltammetry (SV) is a very sensitive and selective electrochemical method to determine the concertation of metal ions in biological samples (Kounaves, 1997). Stripping voltammetry consists of a three-electrode system: a working electrode, a reference electrode and a counter electrode (Figure 2.9) (Pramanik et al., 2013).

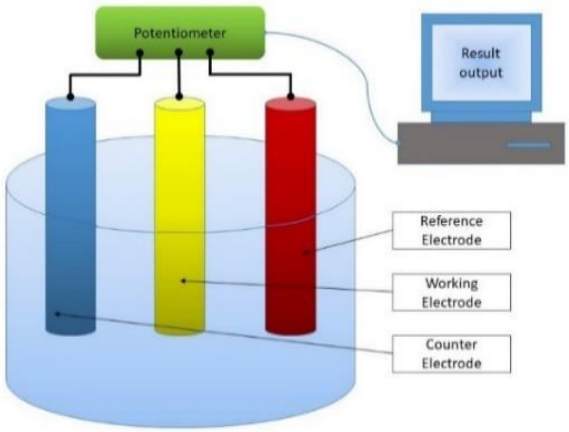

Figure 2.9. Electrode system for Cd measurement (redrawn from Pramanik et al. (2013)).

Mercury electrode has been widely used for electrochemical Cd analysis as the working electrode with Ag/AgCl as the reference electrode and a platinum counter electrode (Shams and Torabi, 2006). However, the toxicity of mercury today excludes its application as the working electrode in stripping analysis and attempts have been made to introduce modified carbon-paste electrodes in stripping analysis of metal ions (Afkhami et al., 2013). Afkhami et al. (2012) reported that phosphorusylide N-BDMP is a suitable modifier for constructing a chemically modified carbon electrode. With this

combination, they successfully measured Cd^{2+} with a lower detection limit of 6.6 µg/L in biological samples (Table 2.12). A diacetyldioxime modified carbon paste electrode was developed by Hu et al. (2003) which showed a 101% Cd^{2+} recovery in water samples. Marinho et al. (2013) developed $(BiO)_2CO_3$ and Bi_2O_3 nanostructure incorporated graphite composite electrodes for the determination of Cd^{2+} using square wave anodic stripping voltammetry. This electrode showed a lower detection limit of 0.65 µg/L for $(BiO)_2CO_3$ modifier and 0.26 µg/L for the Bi_2O_3 modifier. Leoncini et al. (2019) developed a Gold nanoparticle (AuNP) modified graphene carbon paste electrode with a lower detection limit of 267 µg Cd/L to determine Cd^{2+} ions in environmental samples (Table 2.12). Table 2.12 provides an overview of different studies on the development of Cd^{2+} ion-specific electrodes using different modifiers and methods.

Table 2.12. Summary of different Cd^{2+} ion electrodes prepared in different studies.

Modifier	Method	Lower detection limit (µg Cd/L)	Linear range (µg Cd/L)	Reference
Nano-porous pseudo-carbon paste electrode	DPSV	8.7	21-634	Liu et al. (2019)
Spent coffee grounds	ASV	18×10^3	-	Estrada-Aldrete et al. (2020)
Gold nanoparticles (AuNP) modified graphene	CV	267	750-1000	Leoncini et al. (2019)
Lanthanum	SQWAV	0.12	5-500	Ismail et al. (2019)
Zeolite antimony oxide	LSASV	11.23	80-150	Le Hai et al. (2020)
Antimony film modified sodium montmorillonite	SQWAV	0.25	4-150	Chen et al. (2016)
Phosphorous ylide nitro benzoyl di phenyl methylene phosphorane	CV	6.6	10-2000	Afkhami et al. (2012)
N-P chloro phenyl cinnamon hydrxamic acid	CV	1.1	4-7.	Fanta and Chandravanshi (2001)

Note: DPSV-Differential Pulse Stripping Voltammetry; ASV-Anodic Stripping Voltammetry; CV; Cyclic Voltammetry; SQWAV; Square Wave Anodic Stripping Voltammetry; LSASV-Linear Square Wave Anodic Stripping Voltammetry.

2.12 Summary and knowledge gaps

The application of phosphate fertiliser to New Zealand agricultural lands has played a key role in the addition of higher amounts of Cd to the soil. Even though Cd is a non-essential element for plants, studies have found that it is effectively taken up by the root systems of many plant species and is translocated throughout the plant. However, different plants have different mechanisms to uptake Cd from the soil and translocate the metal from roots to shoots. Stafford et al. (2016) recently showed that forage species such as chicory and plantain can accumulate significantly higher Cd concentrations, from even low Cd soils, when compared to grasses and legumes. Furthermore, literature has identified that Cd accumulation in the liver and kidney of grazing lambs is strongly related to daily dietary Cd intake. These studies suggest that a change in pastoral species composition away from ryegrass and clover to forage crops has the potential to increase Cd intake by grazing animals. Therefore, it is important to investigate the mechanisms underpinning Cd uptake and translocation in chicory and plantain to better manage the risk of high Cd accumulation in the offal. There have been no studies published on the Cd uptake mechanisms of common forage species used in New Zealand agriculture.

Available literature suggested that interactions between plant species and chemical changes in the rhizosphere soil play a key role in Cd uptake mechanisms. A clear message from the literature is that the type and concentration of LMWOAs secreted by plant roots vary as a function of plant species and soil Cd concentration. LMWOAs secreted by plant roots may influence plant Cd uptake through forming stable complexes with bioavailable soil Cd^{2+} and could be an *in planta* or *ex planta* Cd detoxification mechanism to alleviate the toxicity of free Cd^{2+} ions via organic complexation. This literature review has identified the need to investigate changes in rhizosphere chemistry which may be

associated with LMWOA secretion by chicory and plantain to better understand plant root Cd uptake mechanisms of these two forage crops.

Root to shoot Cd translocation via the xylem is the main process accounting for Cd accumulation in the aerial parts of plants. Available literature suggests that Cd which enters the root membrane can be translocated as free Cd^{2+} ions via xylem sap towards aerial parts of the plant. The review of literature has emphasised the importance of determining the free Cd^{2+} ion concentration in chicory and plantain xylem sap to better understand the mechanism of Cd translocation in these two forages. Previous literature has indicated that chemically-modified carbon-paste electrodes can be used to determine free Cd^{2+} ion concentrations during environmental sample analysis. However, there are no studies published on the application of chemically modified electrodes to determine the free Cd^{2+} ion concentration in plant/ xylem saps. There is, therefore, good potential to develop a novel electrode to quantify free Cd^{2+} ions in plant xylem sap. Such an electrode could allow direct measurement of low concentrations of free Cd^{2+} ions in low volume solutions that are characteristic of biological samples.

Literature also shows that different soil Cd levels can affect the production of LMWOAs in xylem sap and these produced organic acids can act as potential chelators to facilitate trace metal transport in the xylem sap of plant species. Available literature suggests that the formation of organic metal complexes is an internal detoxification mechanism for Cd. It is therefore important to assess the relative production and translocation of LMWOA in the xylem of chicory and plantain in response to Cd in the growing media.

2.13 Research questions

This literature review has revealed key knowledge gaps that constrain understanding of Cd uptake and translocation mechanisms in chicory and plantain. The overall aim of the research described in this thesis is to determine the plant Cd uptake mechanism in chicory and plantain and this research has been designed to address three knowledge gaps.

The first knowledge gap is the need to understand the effect of increasing Cd levels in growing media on LMWOA secretion by chicory and plantain roots. Specifically, the following research questions have been identified:

- How does the type and concentration of LMWOA secreted by plant roots vary with increasing Cd concentration in the growth media of both plants?
- Is there any difference in LMWOA secretion between chicory and plantain?
- How does root secrete LMWOA impact plant Cd uptake by both plants?
- How do the variations in LMWOA production in both plants explain the differences in plant Cd uptake by chicory and plantain?

Second is the need for knowledge of the chemical speciation of Cd in xylem sap, particularly the free Cd^{2+} ion concentration, as well as quantitative data on the concentration of LMWOA-complexed Cd in chicory and plantain xylem saps. However, quantification of very low free Cd^{2+} concentrations in plant sap is a significant analytical challenge due to the lack of any reliable analytical tools for micro or nano level Cd^{2+} ion measurement.

To satisfy the above objective the following research questions have been identified:

- Can a simple ion-specific electrode quantify low concentrations of free Cd^{2+} ions species in the xylem sap be developed?
- In which chemical form is Cd mainly translocated inside the xylem sap of both plants?
- How does the composition and quantity of LMWOA in the xylem sap vary with increasing Cd concentration in the growth media of both plants?
- Is there any difference in xylem sap LMWOA production between chicory and plantain?
- How does the xylem sap LMWOA impact on shoot Cd concentration of both plants?
- How does the LMWOA production in xylem sap alleviate the Cd toxicity in both plants?

The third knowledge gap relates to how the external application of LMWOA influences the uptake of Cd by chicory. Specifically, the following research questions have been identified:

- Does the external application of different LMWOA impact plant Cd uptake and translocation?
- What is the specific active range of LMWOA concentration responsible for influencing Cd uptake and translocation?
- Does the external application of LMWOA alleviate Cd toxicity in plants?

The research described in this thesis has been designed and conducted to explore and (where possible) answer these research questions.

Chapter 3

Effect of soil Cd on root organic acid secretion by forage crops

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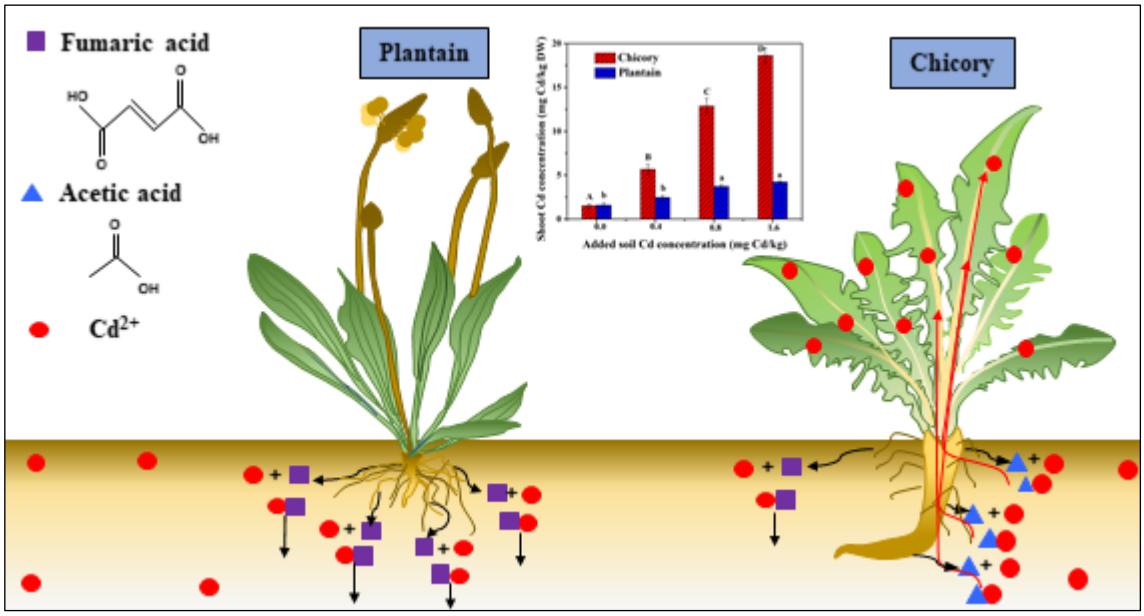
3.1 Graphical Abstract



3.2 Abstract

Two forage species used in New Zealand pastoral agricultural systems, chicory (*Cichorium intybus* L.) and plantain (*Plantago lanceolata* L.), show differential ability to absorb and translocate Cd from roots to shoots. Chicory can accumulate Cd from even low Cd soils to levels that might exceed regulatory guidelines for Cd in fodder crops and food. Plant uptake of Cd is dependent on interactions between the rhizosphere soil-plant root interface. Plants roots secrete LMWOAs into the rhizosphere soil, which can influence Cd uptake. A glasshouse experiment was conducted to evaluate the influence of increasing Cd concentrations on the secretion of LMWOAs by chicory and plantain roots. Chicory and plantain were grown in soil-filled rhizocolumns under increasing Cd levels (0 (Control), 0.4, 0.8 and 1.6 mg Cd/kg soil) for 60 days and showed variable secretion of oxalic, fumaric, malic and acetic acids as a function of Cd treatment. Chicory showed significantly ($P<0.05$) lower secretion of fumaric acid and higher secretion of acetic acid than plantain at all Cd treatments. There was no clear trend of oxalic and malic acid secretion as a function of soil Cd concentration between these two plants. Chicory showed significantly higher ($P<0.05$) shoot Cd concentration compared to plantain at high Cd treatments (i.e. 1.6 mg Cd/kg). Thus, this study suggests that the greater shoot Cd concentration in chicory relative to plantain can be explained by increased acetic acid and reduced fumaric acid secretion in chicory compared to plantain.

3.3 Introduction

Chicory and plantain can accumulate significantly higher Cd concentrations, from even low Cd soils, when compared to grasses and legumes which have traditionally been used in New Zealand agriculture (Stafford et al., 2016). Grazing Cd-rich forage has the

modelled potential to cause an exceedance of the maximum guideline level for Cd in the kidneys and livers of livestock (Lee et al., 1996), although the relative risks of such exceedance between forage crops are poorly understood. Many studies have suggested that interactions between different plant species and chemical changes in their vicinity of plant roots (in the rhizosphere soil) play a key role in Cd uptake mechanisms by plants (Mench et al., 1991; Hinsinger et al., 2006). There is good evidence that plant roots secrete many compounds to the plant root-soil interface, which can modify the physical and chemical characteristics of the rhizosphere zone and influence Cd bioavailability (Hill et al., 2002; Hinsinger et al., 2006). Compounds in root exudates can be categorized as either high molecular weight or low molecular weight materials (Luo et al., 2014). Among these, LMWOAs such as malic, oxalic, acetic, fumaric and citric acids are negatively charged anions that are capable of forming stable complexes with bioavailable Cd^{2+} to influence plant Cd uptake (Han et al., 2006; Zhu et al., 2011). Previous studies on the effect of these LMOWAs in root exudates on bioavailable Cd in soil were discussed in the review literature in Chapter 2. For example, Fu et al. (2018) reported significant ($P<0.05$) positive correlations between oxalic ($R=0.93$) and malic ($R=0.92$) acids and the total tissue Cd concentration for rice cultivar (Lu527-8) grown in hydroponic solutions across a Cd concentration range of 0-5 mg/L. The secretion of LMWOAs by plant roots could be an *in-planta* detoxification mechanism to alleviate the toxicity of free Cd^{2+}ions via organic complexation (Pence et al., 2000; Wei et al., 2007). Several studies have suggested that the secretion of LMWOAs into the rhizosphere can also contribute to *ex-planta* Cd detoxification mechanisms by inducing the formation of metal-organic acid complexes in the rhizosphere soil which immobilize contaminants before they enter the root membrane (Pinto et al., 2008; Zhu et al., 2011). For example, Zhu et al. (2011) found

that root-secreted oxalate plays an important role in reducing the Cd toxicity in tomato by excluding the entry of Cd into the root cell membrane.

The impact of root exudates on Cd uptake by roots deserves more research, particularly for forage species of economic importance to countries such as New Zealand where agriculture is dependent on pastoral grazing systems. Therefore, the objective of the current study was to assess Cd uptake in two forage species (chicory and plantain) as a function of rhizosphere soil chemistry and to compare their physiological response of these forage species to different levels of Cd in soil.

3.4 Materials and methods

3.4.1 Outline of the experiment

The forage species chicory (*Cichorium intybus* L.) and plantain (*Plantago lanceolata* L.) were grown in replicate rhizocolumns containing field soil spiked with three different Cd concentrations representative of the range of Cd concentrations expected in New Zealand pastoral soils. The study was conducted under greenhouse conditions. Plant Cd, LMWOAs concentrations and related parameters were analysed after 60 days of plant growth.

3.4.2 Pot experiment

The pot experiment was set up in a greenhouse at the Massey University Plant Growth Unit with a day/night temperature of 17/20 ℃. A bulk sample of Manawatu recent soil (Dystric Fluventic Eutrudept in the US Soil Taxonomy Classification as reported by Hewitt (2010)) was collected from the top 15 cm of the soil profile at the dairy No 1 farm,

Massey University, Palmerston North (400 22’ 55. 56’’ S 1750 36’ 21. 37’’ E) (Jeyakumar et al., 2010). The Manawatu recent soil had a background Cd concentration of 0.21 mg/kg and was selected for this study due to the low organic carbon content relative to productive New Zealand soils (Table 3.1 and Appendix 1). The collected soil was air-dried at 30 ℃ for 5 days, sieved through a <4 mm sieve. The soils were (1.5 kg each) spiked with a calculated amount of $CdCl_2$ at the rates of (0 (control), 0.4, 0.8 and 1.6 mg Cd/kg) separately to form four different treatments. A bulk sub-soil sample was further sieved through a <2 mm stainless steel sieve and stored for soil characteristic analysis (Table 3.1). Twenty-four pots (2L) were filled with the soil providing 6 pots per treatment and all pots were incubated for one month to equilibrate Cd within the soil matrix. Incubated soil (700 g) was then transferred to a rhizocolumn (Figure 3.1). The rhizocolumn is a polyvinyl tube to support plant growth based on the design concepts of a rhizo-box explained by Wang et al. (2002). The rhizocolumn had two sections, each of each 50 mm height, separated by a nylon mesh (20 microns). The nylon mesh vertically separated the rhizosphere soil from the rest of the soil during the plant growth period. The radius of the top and bottom sections were 40 mm and 50 mm, respectively (Figure 3.1). One viable and healthy seedling of each plant was planted in the middle of the upper section of each column. Chicory and plantain were planted in the four different Cd treatments and replicated three times. The greenhouse experimental set-up was arranged in a Complete Randomised Design (CRD) and maintained at a pot-field capacity of 70% for 60 days in a greenhouse; on average, day/night temperature ranged between 17 and 20 ℃.

Table 3.1. Chemical properties of the Manawatu Recent soil used in this study.

Soil parameter	Values
pH	5.95
TOC (g/kg)	27.7
OM (g/kg)	55.4
Total Cd (mg Cd/kg)	0.21
CEC (meq/100g)	15.85
Sodium extractable Fe (%)	2.61
Sodium extractable Al (%)	0.79
Acid oxalate extractable Fe (%)	1.63
Acid oxalate extractable Al (%)	0.45

3.4.3 Plant harvest and soil sampling

Sixty days after transplanting, plants were carefully removed from the top section of each rhizocolumn. Soil from the top section was collected as two portions: (1) the rhizosphere soil (R) which was soil adhered to roots and which was collected by gently scraping the soil by hand, and (2) the bulk soil (B) which was soil attached loosely to the roots and which was collected by shaking the roots carefully (Figure 3.1) (Jeyakumar et al., 2014; Xin et al., 2015). The bottom section soil was cut into 3 layers starting from the top of the section using a knife (from next to mesh), as shown in Figure 3.1. The first, second and third layers were labelled as 1 mm near rhizosphere (S1), 2 mm near rhizosphere (S2) and 3 mm near rhizosphere (S3), respectively (Figure 3.1). All soil samples were air-dried and ground to pass through a 2 mm sieve and stored in sealed plastic bags at room temperature until analysis.

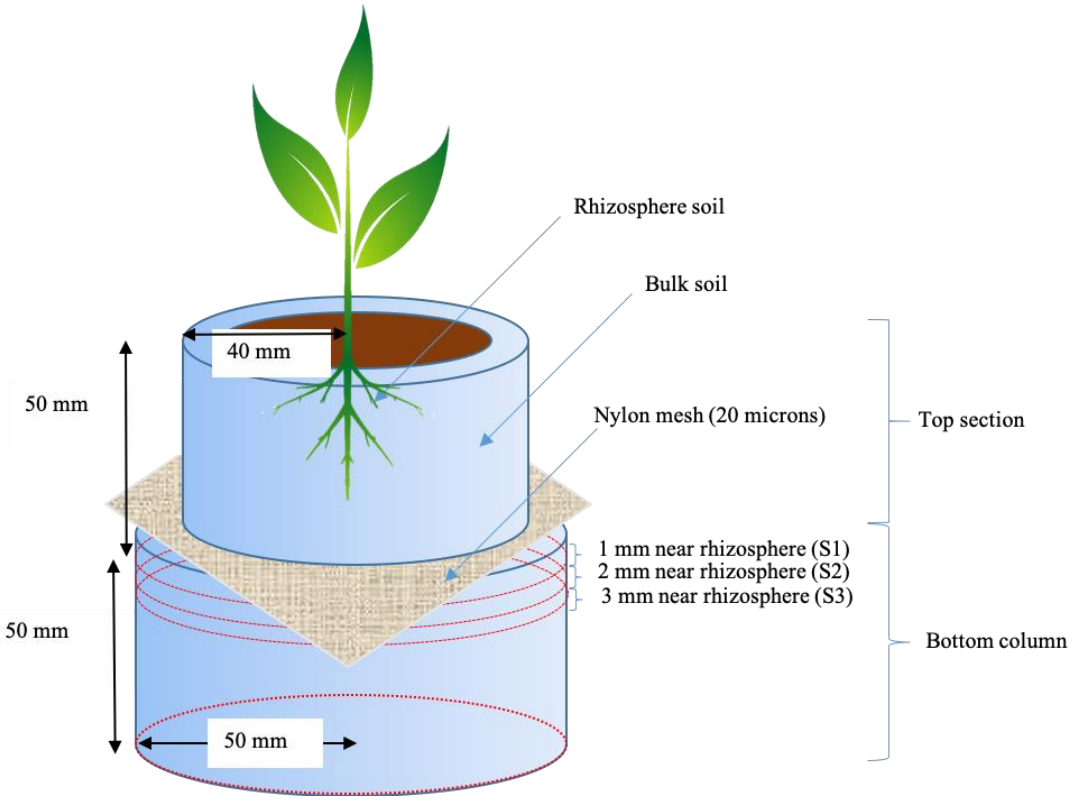


Figure 3.1. Rhizocolumn used in the greenhouse experiment (Diagram not to scale). Soil adhered to the root and the soil loosely bound to root in the top section was defined as the rhizosphere and bulk soil, respectively. The soil under the nylon mesh in the bottom section was defined as near rhizosphere soil.

3.4.4 Collection of root exudates from plantain and chicory

Plant root exudates were collected immediately after harvest using the procedure explained by Bao et al. (2011). After the bulk and rhizosphere soil was removed, the uprooted plant roots were carefully washed with de-ionized water and transferred to Erlenmeyer flasks containing 200 mL de-ionized water. The flasks were covered with aluminium foil and the root exudate was collected under greenhouse conditions (day/night temperature of 17/20 °C) with the solution aerated using aquarium pumps to maintain aerobic conditions. The solutions obtained during the first hour of the collection were not used for analysis and were discarded to remove exudates from possibly injured

root cells during harvest and washing. The plants were then again allowed to secret into fresh de-ionized water for 4 hrs following the same procedure. The solution was filtered through a 0.45 µm filter and freeze-dried for LMWOA analysis.

3.4.5 Plant analysis

3.4.5.1 Plant biomass

Plant shoots and roots were separated at harvest and dried at 60 ℃ to constant weight. The total dry weight of each plant portion was recorded. Dry shoot and root biomass were finely ground using a Cyclotech mill and stored for further chemical analysis.

3.4.5.2 Plant tissue total Cd concentration

For each shoot and root plant biomass sample, 0.1 g of dried and ground material was digested with conc HNO_3 (10 mL) and diluted to 25 mL with de-ionized water. The total Cd concentration in the digest solutions was quantified using graphite furnace atomic absorption spectrometry (GFAAS) (Perkin Elmer 900z, Germany). The shoot to root translocation factor (TF=shoot Cd concentration: root Cd concentration) was calculated for each Cd treatment of both plants (Jeyakumar et al., 2014).

3.4.5.3 HPLC analysis for LMWOAs in root exudates

The composition and concentration of LMWOAs in root exudates were analysed by High-Performance Liquid Chromatography HPLC (Dionex, Thermo Fisher Scientific, Australia) as described by Cawthray (2003) with minor modifications. The freeze-dried exudate samples were dissolved with 4 mL of the HPLC mobile phase solution 25 mM KH_2PO_4. The mixtures were filtered through 0.22 µm filters to remove suspended

material prior to injection into the HPLC. The separation was conducted on a 250 × 4.6 mm (5 µm particle size) C_{18} reverse-phase column. The sample solutions (100 µL) were injected into the column with a flow rate of 1.0 mL/min at 25°C and UV detection at 210 nm. Potassium dihydrogen phosphate (25 mM) solutions were used for isocratic elution. Identification of organic acids was performed by comparing retention times in root exudate samples with those retention times obtained by analysing a standard mixture including seven organic acids (i.e. acetic, citric, fumaric, malic, oxalic, succinic, and tartaric), which are common in root exudates (Cawthray, 2003). Standard mixture solutions of increasing concentration (i.e. 20, 40, 60, 80, 100, 120, 140, 180, 200 mg/L) of the same seven LMWOAs were prepared to determine the concentration of organic acids in the root exudate samples. Data are presented as mg LMWOA/kg plant root dry weight (DW).

3.4.6 Soil analysis

The pH of soil samples collected at harvest was measured (1: 2.5 w/w soil: water ratio) using a Eutech Instruments Cyber Scan pH 310. Briefly, 5 g of soil was weighed into the pH cup and 12.5 mL of deionised water was added. Samples were stirred vigorously for at least one minute, before being left to stand overnight. On the following day, the pH of the soil samples was quantified. For bioavailable soil Cd concentration, (rhizosphere, bulk soil and near rhizosphere zone) 8 mL $MgCl_2$ (1 M, pH 7) was added to 1 g of soil in a centrifuge tube and shaken in an end-over-end shaker for 1 hour at room temperature. The solution was centrifuged at 13,000 g for 30 min and filtered through Whatman 42 filter paper. The filtered solution was then analysed using GFAAS (Perkin Elmer 900z, Germany).

3.4.7 Quality control measures

All chemicals used in the experiments were of analytical grade. The limit of detection for Cd in this work was 0.002 mg Cd/L. The accuracy of the measurements was assessed by analysing certified reference materials in parallel with unknown samples. For total Cd concentration, CRM 051-050 clay 2 sample, a soil from western USA (total Cd 42.2 mg Cd/kg), was used as the certified reference material. The mean Cd concentration of the CRM 051-050 was obtained as 43.7±3 mg Cd/kg, which is 96-110% of the expected value. For plant total tissue Cd analysis, NIST 1573a (National institute of standards and technology, tomato leaves-1.52 mg Cd/kg) was used as certified reference material and found to be within 94-108% of the expected mean value.

3.4.8 Statistical analysis

Statistical analysis was conducted with Minitab 18 and OriginPro 9 (OriginLab, USA) statistical software. The effect of Cd treatments on different plant and soil variables was statistically analysed using a one-way ANOVA test; if a significant ($P<0.05$) main effect was detected, the difference between treatment means was tested using a Tukey HSD posthoc test. The significant differences of each LMWOA concentration between chicory and plantain were tested using an unpaired t-test.

3.5 Results and discussion

3.5.1 Soil pH

Many studies have reported pH variations between the rhizosphere and bulk soil when different plant species are exposed to potentially toxic elements such as Cd (Stoltz and Greger, 2002; Blossfeld et al., 2010; Tanwir et al., 2015). In the present study, the pH of the rhizosphere soil was significantly lower than the bulk soil for all treatments except the 1.6 mg Cd/kg soil treatment for plantain with the difference in the range of 0.3-0.5 pH units (Table 3.2). This result was similar to a significantly reduced pH (5.94) in the rhizosphere relative to the bulk soil (pH 6.4) reported for mangrove plants grown in sediment with a Cd concentration range from 5-50 mg Cd/kg (Jingchun et al., 2008). Séguin et al. (2004) proposed that increased LMWOAs secretion into the rhizosphere soil due to Cd-induced plant stress may reduce rhizosphere pH compared to the bulk soil pH. LMWOAs are mainly in a dissociated form in the plant cytosol and play a key role in buffering the cytosolic pH. They are predominantly secreted as anions to rhizosphere soil, leading to a cation-anion imbalance inside the plant cytosol. To balance this, plants efflux H^+ ions from plant cells (plant cytosol) into the rhizosphere soil from the proton pump, and this contributes to a decrease of rhizosphere pH (Tanwir et al., 2015; Luo et al., 2018). For the current study, the rhizosphere and bulk pH values for chicory and plantain were independent of the added soil Cd concentration. The discrepancy between literature reported trends and the observations of the current study may be due to the relatively low soil Cd concentrations used in the study. Zeng et al. (2008) also observed a non-significant difference of pH at relatively low Cr levels (<2.5 mg Cr/L) in a rice-growing nutrient solution.

Table 3.2. pH of the rhizosphere and bulk soils of chicory and plantain at harvest.

Added soil Cd concentration (mg Cd/kg)	Soil pH			
	Chicory		Plantain	
	Rhizosphere	Bulk	Rhizosphere	Bulk
Control (0)	5.63±0.05b	5.90±0.05a	5.70±0.06b	5.92±0.04a
0.4	5.35±0.03b	5.73±0.05a	5.61±0.04b	6.05±0.02a
0.8	5.61±0.07b	5.91±0.02a	5.53±0.09b	5.73±0.01a
1.6	5.37±0.04b	5.89±0.04a	5.75±0.09a	5.84±0.02a

Data are means±standard errors of three replicates. Values in each line, followed by the same letter within a row for each plant are not significantly different at $P<0.05$ (n=3).

3.5.2 The bioavailable Cd concentration in soil

The concentration of bioavailable Cd in the rhizosphere (R) and bulk (B) soil (top section soil) and from all intervals of the near rhizosphere soil (S1, S2 and S3), increased with increasing Cd treatment level for both plants (Table 3.3). A difference in bioavailable Cd concentration between the rhizosphere and near-rhizosphere compartments was observed for three treatments (1.6 mg Cd/kg treatment for both plants and the 0.4 mg Cd/kg treatment for plantain) although this variation was insufficient to define an overall significant difference in bioavailable Cd concentration between the rhizosphere and near rhizosphere soil with increasing soil Cd concentration (Table 3.3). Further analysis was therefore not conducted of the near rhizosphere soil layers. The bioavailable Cd concentration was significantly ($P<0.05$) lower in the rhizosphere soil than in the bulk soil for both plants and all Cd treatments (Table 3.3). Previous studies have shown that the bioavailable Cd concentration of soil will increase as a function of changes in soil pH, with the rhizosphere exhibiting reduced pH due to the secretion of LMWOAs (Haoliang et al., 2007; Chen et al., 2011). However, in the present study, despite the significantly lower pH observed in the rhizosphere soil compared to the bulk soil (Table 3.2), the bioavailable Cd concentration was significantly lower in the rhizosphere soil than the bulk soil for all Cd treatments in both plants (Table 3.3). This discrepancy might be explained through higher plant uptake of Cd from the rhizosphere soil than the bulk soil.

Ru et al. (2006) reported that at a soil Cd concentration of 60 mg Cd/kg, the exchangeable Cd concentration (DTPA-extractable Cd) in the rhizosphere soil (31.5 mg Cd/kg) was significantly ($P<0.05$) lower than that in non-rhizosphere soil (38.5 mg Cd/kg) for Indian mustard. These authors suggested that plants may take up more Cd from near the root and that removed Cd is not quickly replenished from soil further away from the roots.

Table 3.3. Bioavailable Cd concentration of rhizosphere, bulk and near rhizosphere soil layers for chicory and plantain.

	Bioavailable Cd concentration (mg Cd/kg)			
Soil Layer	**Control**	**0.4 mg Cd/kg**	**0.8 mg Cd/kg**	**1.6 mg Cd/kg**
		Chicory		
Rhizosphere	0.084±0.005b	0.272±0.011b	0.556±0.027b	1.060±0.025c
S1	0.117±0.001a	0.339±0.016ab	0.686±0.051ab	1.280±0.038ab
S2	0.101±0.003ab	0.335±0.018ab	0.642±0.010ab	1.324±0.003a
S3	0.102±0.001ab	0.353±0.020b	0.649±0.004ab	1.292±0.020a
Bulk	0.131±0.002a	0.344±0.006ab	0.759±0.056a	1.178±0.007b
		Plantain		
Rhizosphere	0.086±0.006b	0.360±0.032c	0.671±0.003ab	1.024±0.039c
S1	0.146±0.023ab	0.451±0.016b	0.635±0.014b	1.240±0.017b
S2	0.155±0.011ab	0.440±0.003bc	0.663±0.012ab	1.424±0.025a
S3	0.180±0.001a	0.585±0.003a	0.677±0.011ab	1.447±0.017a
Bulk	0.155±0.028ab	0.414±0.013bc	0.702±0.013a	1.438±0.019a

Data are mean±standard error of three replicates. Values in each line, followed by different alphabetic letters within a column, for each plant, are significantly different at $P<0.05$ (n=3). Results are reported to three decimal points based on the limit of detection for the GFAAS of 0.002 mg Cd/L.

3.5.3 Composition and concentration of LMWOAs in root exudates

The primary route of LMWOA production is inside cell mitochondria as an intermediate step of photosynthesis through a plant's tricarboxylic acid cycle (TCA) (Igamberdiev and Eprintsev, 2016). LMWOAs are directly and indirectly involved with many metabolic processes such as the regulation of cytosolic pH and the balancing of charges during excess cation uptake (Hinsinger et al., 2003). Fu et al. (2018) suggested that trace metal stress, such as that induced by Cd in the soil, can destabilize the plant cytosolic pH and activate various enzymes in the plant TCA cycle to increase LMWOA production and root secretion (Rengel, 2002). Haoliang et al. (2007) suggested that increasing levels of

trace metal in the soil can cause oxidative stress in plants by depleting antioxidative systems and activating various enzymes in the plant TCA cycle, which also increase LMWOA production in cells. Studies have shown variations of LMWOA secretion by plant roots as a function of soil Cd concentration (Bao et al., 2011; Li et al., 2014; Xin et al., 2015; Montiel-Rozas et al., 2016). For example, Xin et al. (2015) reported that the secretion of oxalic and succinic acids in hot pepper (variety JFZ) increased by around 100 and 33%, respectively, when the Cd concentration in hydroponic solution increased from 0 (control) to 0.2 mg Cd/L. In the current study, the composition and quantity of LMWOAs secreted by chicory and plantain varied as a function of the added soil Cd concentration (Table 3.4). Oxalic, fumaric, malic and acetic acids secreted by chicory, and oxalic, fumaric and malic acids secreted by plantain, were the major LMWOAs analysed for all Cd treatments.

The concentration of oxalic acid secreted by chicory and plantain did not significantly ($P>0.05$) change with increasing soil Cd levels, although there was a nominal increase by 6% and 24%, respectively, at the 1.6 mg Cd/kg soil level compared to the control (Table 3.4). The concentration of acetic acid secreted by chicory showed a nominal decrease of 50% with increasing soil Cd concentration (Table 3.4). The concentration of malic and fumaric acids secreted by chicory did not significantly differ between the control, 0.4 and 0.8 mg Cd/kg treatments, but significantly increased ($P<0.05$) by 76% and 140%, respectively, at the 1.6 mg Cd/kg treatment relative to the control (Table 3.4). For plantain, the concentration of fumaric acid (80.3-242.4 mg/kg root DW) and malic acid (73.1-302.7 mg/kg root DW) did not show any trend with the increasing concentration of Cd in the soil (Table 3.4). Many studies have shown the formation of Cd-LMWOA complexes enhance Cd uptake by plants (Chen et al., 2003; Hawrylak-Nowak et al., 2015; Mnasri et al., 2015) and such studies suggest that Cd chelation with LMWOAs may create

an important pathway to mitigate the toxicity of free reactive metal ions inside the plant (Pence et al., 2000; Wei et al., 2007). Han et al. (2006) reported that Cd can complex with organic acids to produce mobile and soluble organically bound Cd complexes, which can penetrate the lipid membrane of root cells and act as a major contributor for Cd uptake by maize (Adeleke et al., 2017). Li et al. (2014) suggested that Cd-organic acid complexes might enter plants through breaking the endodermis and Casparian strips of the root cells. Ehsan et al. (2014) reported that organometallic complexes in the soil solution can act as a carrier for Cd^{2+} ions towards the root surface and that these complexes can disassociate into free Cd^{2+} at the root surface which can be absorbed by the root membrane.

Table 3.4. The concentration of LMWOAs (mg acid/kg root DW) secreted from the roots of chicory and plantain growing under increasing soil Cd concentrations.

Plant species	**Added soil Cd Concentration**			
	0 (control)	**0.4 mg Cd/kg**	**0.8 mg Cd/kg**	**1.6 mg Cd/kg**
		Oxalic acid		
Chicory	201.9±27.8aA	169.8±14.1aA	159.9±30.1aA	214.5±4.2aA
Plantain	192.3±28.2aA	152.8±15.1aA	131.5±35.1aA	238.8±21.4aA
		Malic acid		
Chicory	165.7±21.5aB	272.2±18.6aAB	264.7±28.7aAB	291.2±30.3aA
Plantain	256.7±43.2aA	73.1±14.7bB	302.7±35.9aA	110.1±22.9bB
		Fumaric acid		
Chicory	5.2±0.2bB	4.7±0.1bB	3.6±0.3bB	12.5±1.9bA
Plantain	162.4±25.0aAB	92.9±16.2aB	242.4±28.4aA	80.3±10.6aB
		Acetic acid		
Chicory	150.1±41.4A	114.3±13.8A	107.2±18.5A	100.1±0.9A
Plantain	ND	ND	ND	ND

Data are mean±standard error of three replicates. Values followed by different small alphabetic letters within a column for each LMWOA are significantly different between the two plants at $P<0.05$ (n=3). Values followed by different CAPITAL alphabetic letters within a row for each LMWOA are significantly different among Cd treatments in a plant at $P<0.05$ (n=3). Note: ND-Not Detected.

3.5.4 Biomass dry matter content and translocation of Cd in plant tissues

There was no significant effect (P>0.05) of increasing soil Cd concentration on the shoot biomass of chicory and plantain, or the root biomass of plantain. There was a significant (P<0.05) effect of Cd on the root biomass of chicory, which increased for all Cd treatments relative to the control (Table 3.5). This observation is in agreement with previous studies where, for some plants, low concentrations of soil contaminants can stimulate the activity of RNA and protein synthases, which promote plant growth (Chi et al., 2019; Kazemi Movahed, 2020).

Table 3.5. Root and shoot dry weights of chicory and plantain.

Added soil Cd concentration (mg Cd/kg)	**Plant dry weight (DW) (g)**			
	Chicory		**Plantain**	
	Root	**Shoot**	**Root**	**Shoot**
Control (0)	0.14±0.01b	0.25±0.04a	0.33±0.03a	0.28±0.01a
0.4	0.29±0.01a	0.31±0.02a	0.30±0.03a	0.32±0.01a
0.8	0.26±0.03a	0.29±0.02a	0.28±0.01a	0.29±0.03a
1.6	0.33±0.03a	0.33±0.01a	0.25±0.01a	0.33±0.02a

Data are mean±standard error of three replicates. Values in each line, followed by different alphabetic letters within a column for each plant are significantly different at P<0.05 (n=3).

There was a significant increase (P<0.05) in the Cd concentration of roots and shoots of both plant species as a function of the soil Cd concentration (Figure 3.2). The chicory shoot Cd concentration increased from 1.5 to 18.6 mg Cd/kg DW and the root Cd concentration increased from 1.5 to 4.2 mg Cd/kg DW as the soil treatment concentration increased from 0 to 1.6 mg/kg. For plantain, the shoot Cd concentration increased from 1.6 to 4.2 mg Cd/kg DW and the root Cd concentration increased 0.9 to 10.8 mg Cd/kg DW for the same increase in soil Cd (Figure 3.2). This increase may be due to the significant increase of bioavailable Cd concentration in the rhizosphere soil (Table 3.3) which was significantly (P<0.001) and positively correlated with the shoot and root Cd

concentration of both chicory (R=0.989 and R=0.917, respectively) and plantain (R=0.925 and R=0.882, respectively). Although the shoot Cd concentration increased as a function of soil Cd levels for both plants, the shoot Cd concentration of chicory was a factor of 3.4 greater than plantain at the 1.6 mg Cd/kg treatment. This is in agreement with Abe et al. (2008) who investigated Cd uptake of 93 plants, including chicory and plantain, grown in sandy loam soil (3 mg Cd/kg soil) and who recorded the highest shoot Cd concentration (77 mg Cd/kg DW) in chicory. Stafford et al. (2016) found that chicory had the highest mean tissue Cd concentration of all tested forage species used in New Zealand livestock grazing systems.

The Cd translocation factor (TF) for chicory and plantain, defined as the ratio of the Cd concentration in shoots to roots, was calculated to better explain the relative ability of these plants to translocate Cd from roots to shoots (Mattina et al., 2003). The Cd concentration in chicory shoots for all treatments was higher than in the roots, and the TF increased from 1.0 to 4.4 as soil Cd increased. However, this increase was significant only when the soil Cd concentration increased from 0.4 to 0.8 mg Cd/kg soil (Figure 3.2a). Plantain had a higher Cd concentration in the roots (except for control) than in the shoots and the translocation factor for plantain decreased from 1.9 to 0.4 as the soil Cd concentration increased. This decrease was significant when the soil Cd concentration increased from control to 0.4 mg Cd/kg soil (Figure 3.2b). These results are in agreement with a field study conducted by Sekara et al. (2005) who reported chicory to have the third-highest TF (1.43) of nine plant species grown in a sandy loam soil with a Cd concentration of 1.81 mg Cd/kg soil.

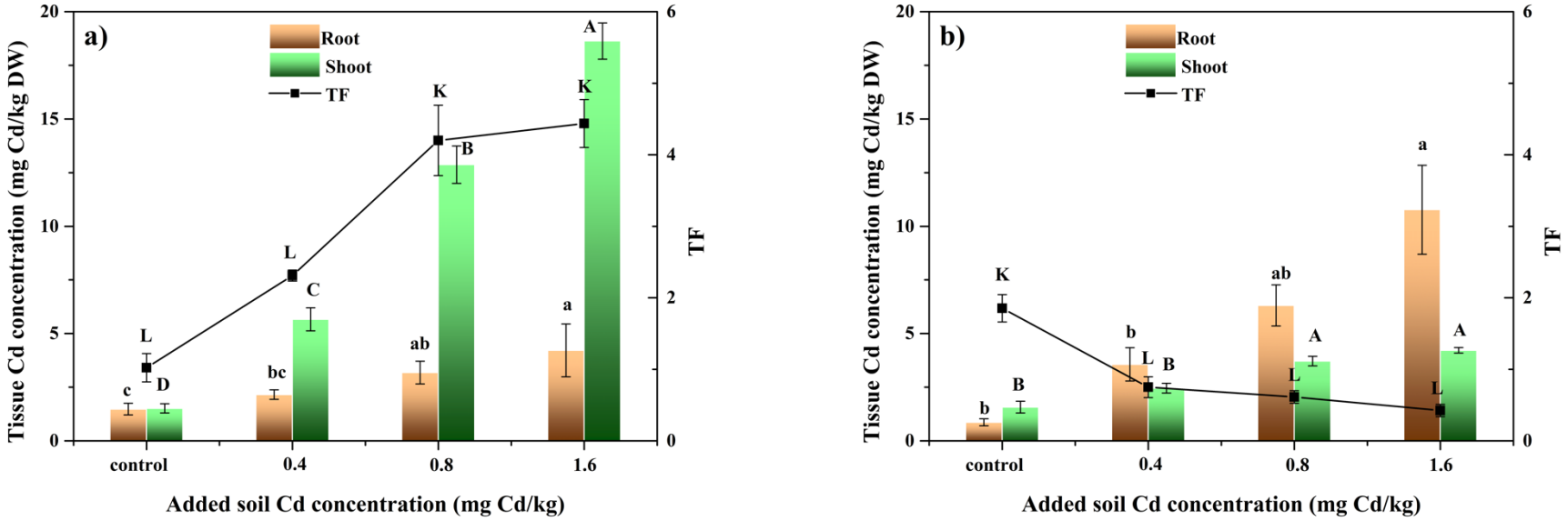


Figure 3.2. Tissue Cd concentration and TF of (a) chicory (b) plantain grown in different soil Cd treatments. Vertical error bars represent ±SE (n = 3). Significant differences between root and shoot tissue Cd concentrations between Cd treatments are represented by Small and CAPITAL alphabet letters, respectively. Values in TF lines followed by different alphabet letters (K and L) are significantly different at P<0.05.

3.5.5 Relationship between LMWOAs concentration and tissue Cd concentration

Experimental growth conditions can influence the development and size of roots, which can then affect the excretion of organic acids (Montiel-Rozas et al., 2016). The significant increase of malic and fumaric acid concentration secreted by chicory at the 1.6 mg Cd/kg treatment relative to the control can be an effect of the significantly greater root biomass at this treatment.

The data of this study suggest that the primary cause for the significant increase of shoot and root Cd concentration in both plants, as a function of treatment level, is the significantly greater bioavailable Cd concentration in rhizosphere soil at the higher soil Cd treatment levels. However, the results of this study propose that the significantly higher shoot Cd concentration in chicory relative to plantain can be explained in terms of variations of LMWOA secretion in chicory. The results of this experiment show significantly less fumaric acid secretion by chicory relative to plantain for all Cd treatments. Fumaric acid is a dicarboxylic acid and has the greatest affinity towards Cd^{2+} ions (fumaric acid-pKa1=3.02, pKa2=4.44) (Adeniji et al., 2010). Several studies have reported that the secretion of fumaric acid by roots significantly reduces plant Cd uptake. For example, Fan et al. (2016) suggested that a significant ($P<0.05$) increase of fumaric acid secretion (by 60%) in rice (cultivar Hua-Hang-Si-Miao) exposed to a Cd+Si (5 mg Cd/L+42 mg Si/L) treatment influenced the chelation of Cd^{2+} ions and reduced plant Cd uptake relative to the control. Kazemi Movahed (2020) found that greater secretion of LMWOAs including fumaric acid (11-fold increase) by soya bean (cultivar AC Hime) at a treatment concentration of 3.3 mg Cd/L reduced Cd bioavailability and uptake by plants relative to the control through the formation of Cd-organic acid complexes in soil.

In this work, secretion of acetic acid was only recorded for chicory at all Cd treatments. Several studies have reported a relationship between acetic acid secreted by roots and plant Cd uptake. For example, Han et al. (2006) showed that Cd uptake by maize plants increased by 110% when the acetic acid concentration in the hydroponic solution increased from 0-15 mg/L (Cd solution concentration 0.56 mg/L). They proposed that mobile and soluble organically bound Cd complexes (acetic-Cd) can easily penetrate cell membranes to increase Cd uptake. Cieśliński et al. (1998) reported that the significant increase ($P<0.05$) of acetic acid secretion (163%) from a high Cd accumulating wheat cultivar (Kyle) relative to a low Cd accumulating wheat cultivar (Arcola) could explain 33% greater Cd uptake in Kyle than Arcola from Sutherland sandy loam soil with a total Cd concentration of 0.41 mg/kg.

This study proposes that the differential response of Cd uptake between chicory and plantain can be explained in terms of specifically fumaric and acetic acid secretion. There was no clear trend of oxalic and malic acid secretion as a function of soil Cd concentration between these two plants the data and cannot implicate these LMWOAs in accounting for differences in Cd uptake between the two species. The data supports that the greater Cd concentration in chicory relative to plantain can be explained by increased acetic acid and reduced fumaric acid excretion. This study proposes that acetic acid in the rhizosphere promotes Cd uptake, while fumaric acid complexes with free Cd^{2+} ions in the soil solution and reduced the potential for uptake. It was concluded that further experiments are essential to substantiate this explained mechanism, and therefore, the experiments were performed with the exogenous application of different LMWOA concentrations and discussed in Chapter 6.

However, management of the risk of high Cd accumulation by chicory and plantain are underpinned by an understanding of the mechanisms of Cd uptake by roots as well as Cd translocation via xylem sap. Therefore, the next chapter will evaluate the mechanism of Cd translocation.

3.6 Summary

The results of this experiment showed that the composition and concentration of LMWOA in root exudates of chicory and plantain varies as a function of Cd treatment in the soil. There was a significantly ($P<0.05$) higher shoot Cd concentration observed in chicory than plantain at higher Cd treatments (i.e. 1.6 mg Cd/kg). Chicory secreted significantly lower fumaric acid concentration and higher acetic acid concentration than plantain. Oxalic and malic acid secretion did not show a clear trend as a function of soil Cd concentration between these two plants suggesting that oxalic and malic acid secretion may not support to explain the differences in Cd uptake between the two species. Therefore, the key finding of this experiment is that the greater Cd uptake in chicory relative to plantain can be explained by increased acetic acid and reduced fumaric acid secretion in chicory compared to plantain. These findings will be further discussed and confirmed in Chapter 6.

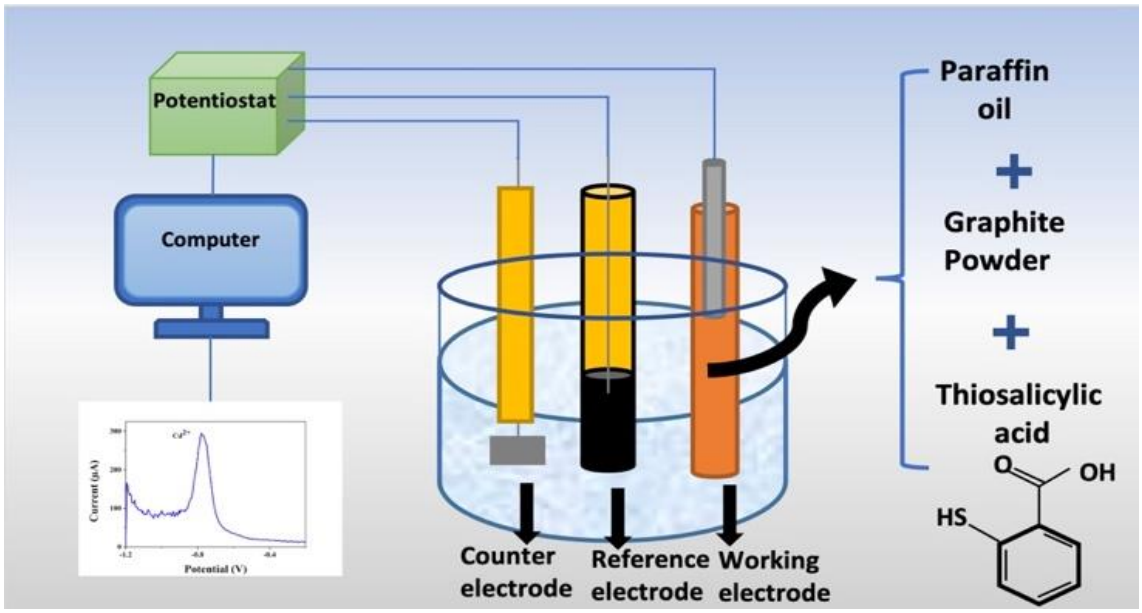
Chapter 4

Development of a thiosalicylic acid-modified ion-specific electrode to explore plant cadmium processes

Part of the results of this chapter was presented at the 15th International Conference on the Biogeochemistry of Trace Elements (ICOBTE) in China. Citation:

- Ubeynarayana, N., Jeyakumar, P., Bishop, P., Pereira, R. C., Anderson, C.W.N., 2019 Development of a thiosalicylic acid-modified ion-specific electrode to explore plant cadmium processes, in "International Conference on the Biogeochemistry of Trace Elements ICOBTE 2019" conference held in Nanjing, China 5-9 May 2019.

4.1 Graphical Abstract



4.2 Abstract

Understanding the mechanisms of Cd uptake and translocation by plant species requires knowledge of the free Cd^{2+} ion concentration in xylem saps. However, the determination of low concentrations of free Cd^{2+} ions in the low volume of xylem saps poses an analytical challenge. In this work, we describe the development and testing of an ion-selective electrode that can measure a low concentration of free Cd^{2+} ions in xylem sap. A modified carbon-paste electrode using thiosalicylic acid (15% w/w) as the modifier is shown to have a detection limit of 11 µg Cd/L (0.1×10^{-6} mol Cd/L) with a high ability to distinguish between total Cd and free Cd^{2+} in samples. The modified electrode measured the free Cd^{2+} ion concentrations in a range of environmental media, including xylem saps with a high precision (RSD<5%).

4.3 Introduction

Efforts to manage the risk of high Cd accumulation by chicory and plantain are underpinned by an understanding of the mechanisms of Cd uptake by roots as well as Cd translocation via xylem sap. Increased understanding of the Cd translocation mechanisms in plants requires knowledge of the chemical speciation of Cd in xylem saps. Research has shown that Cd exists in xylem saps as the free Cd^{2+} ion or Cd complexed with various organic and inorganic compounds (Ueno et al., 2008; Fu et al., 2019). Complexed Cd species can be isolated from xylem saps using advanced separation techniques such as gel exclusion and high-performance liquid chromatography (Kato et al., 2010). However, quantification of very low free Cd^{2+} concentrations in xylem sap is a significant analytical challenge due to the lack of reliable analytical tools for micro or nano level Cd^{2+} ion measurement. Cadmium ion analysis can be carried out with various methods such as

atomic absorption spectrometry (AAS), inductively coupled plasma atomic emission spectrometry (ICP-AES) and microwave plasma atomic emission spectrometry (MP-AES) (Baffi et al., 2002) and even though these methods provide accurate and precise results, they don't have the ability to distinguish between total Cd and free Cd^{2+} in the solution. Instead, these techniques measure total Cd concentration in samples (Hu et al., 2003). Research has established that electrochemical stripping voltammetry techniques such as square wave anodic stripping voltammetry (SWASV), cyclic voltammetry (CV), and linear sweep anodic stripping voltammetry (LSASV) can analyse free Cd^{2+} ions in solution (Ping et al., 2011; Shan et al., 2015). Traditionally, the mercury hanging drop electrode has been used to determine Cd^{2+} ions by such techniques due to the proven reproducibility of analysis which is a function of the purity of the electrode surface (Tanaka et al., 1956). However, the toxicity of mercury and international efforts to ban its use through the Minamata Convention on Mercury has forced researchers to look for alternative options to this electrode. Recently, chemically modified carbon-paste electrodes have found application in metal ion detection, including Cd^{2+} ions in solutions (Ping et al., 2011). A chemically modified carbon paste electrode can be defined as an electrode which is made from a mixture of conducting graphite powder and a modifier (chelator) which has a specific affinity towards a target metal ion (Ping et al., 2011; Chen et al., 2016). Such electrodes have important qualities such as easy fabrication and renewal, low cost, and low background current (Švancara et al., 2009). However, obtaining micro-level detection limits remains a key limitation, especially for Cd^{2+} ions in xylem sap and soil solution.

Simple carbon paste electrodes do not have an affinity towards Cd^{2+}; however, the detection limit of a plain carbon electrode for Cd^{2+} ions can be lowered through modification with specific chelates with a high affinity towards Cd^{2+} ions (Roa et al.,

2003). Le Hai et al. (2020) constructed a chemically modified carbon paste electrode with antimony oxide and determined a Cd detection limit to 11.2 μg Cd/L (0.1×10^{-6} mol Cd/L). A Gold nanoparticle-modified carbon-paste electrode developed by Leoncini et al. (2019) showed a lower detection limit of 267 μg Cd/L (2.4×10^{-6} mol Cd/L); however, the calibration curve was linear only from 750 to 1000 μg Cd/L ($6.7\text{-}8.9 \times 10^{-6}$ mol Cd/L). Several authors have reported that thiosalicylic acid has shown a particularly high affinity towards metal ions, including Cd^{2+} ions (Gismera et al., 2003; Bhowon et al., 2017). Thiosalicylic acid is a readily available commercial off-white solid, which is stable to air, making it a conveniently handled ligand (Wehr-Candler and Henderson, 2016) and it, therefore, may be a potential candidate to develop a Cd^{2+} ion-specific electrode with a lower detection limit compared to previously reported studies.

This chapter reports the development and evaluation of a novel carbon paste electrode modified with thiosalicylic acid which can be used for reliable measurement of free Cd^{2+} ions in xylem saps and soil solutions. The hypothesis of the study is that modification of carbon paste with thiosalicylic acid will yield an electrode with a sufficiently low detection limit to be used in analysing Cd in environmental media. The specific objectives of the study were to (a) define the best experimental conditions for quantification of free Cd^{2+} ions using the developed electrode, and (b) evaluate the technical performance of the developed electrode for assessing free Cd^{2+} ions in environmental solutions.

4.4 Materials and methods

4.4.1 Reagents and solutions

All reagents used were of analytical grade. Thiosalicylic acid (97%) and spectrographic graphite (<20 μm) were obtained from Sigma (USA). Thiosalicylic acid was characterised by Fourier transform infrared spectroscopy (FTIR) technique using a Fourier transform infrared spectrophotometer (Thermo Scientific, Model Nicolet iS5, USA), operating in the region from 4000 to 500 cm^{-1}. Cadmium nitrate and other metal nitrates and reagents were AAS grade. The water used in all experiments was ultrapure with resistivity not less than 18.2 MΩ.cm generated using a Millipore Milli-Q system. Sodium Acetate (0.1 mol/L CH_3COONa) buffer was made by dissolving 0.82 g of sodium acetate (BDH chemicals) and 0.57 mL of glacial acetic acid in 100 mL of Milli-Q water.

4.4.2 Preparation of the modified carbon paste electrode

Voltammetric measurements were performed with a three-electrode system, comprised of a working electrode of thiosalicylic acid modified carbon paste (TSA-CP), a counter electrode of the platinum plate, and a reference electrode of Ag/AgCl (saturated KCl). The modified electrode was prepared by mixing thiosalicylic acid, high-quality graphite powder, and paraffin oil. The mixture was manually homogenised for 20 min using a mortar and pestle, then packed into a PTFE tube (outer diameter: 8 mm; inner diameter: 6 mm) with a stainless-steel rod to make the modified carbon paste electrode (modified electrode). Graphical details of the electrode preparation are presented in Appendix 2.

Voltammetric measurements were carried out using a Shield VO.1 R2 potentiostat attached to a personal computer and controlled by Rodeostat Web-App software. All measurements were carried out in a glass cell (25 mL) at constant room temperature.

4.4.3 Voltammetry responses of TSA-CP electrode - Preliminary Study

Preliminary experiments were carried out to characterise the behaviour of Cd^{2+} ions on the carbon paste electrode modified with thiosalicylic acid (10%, w/w). A carbon paste (CP) electrode (before modification) was used as a reference working electrode, and the voltammogram for 50 µg Cd/L Cd^{2+} ion solution was compared with the modified electrode. Measurement was conducted in 0.1 mol/L CH_3COONa as the supporting electrolyte (pH 4.5), with a pre-concentration time of 600 s and 5 Hz sample rate. A well-defined and strong anodic current peak was observed for a Cd^{2+} ion concentration of 50 µg Cd/L and therefore this Cd^{2+} ion concentration was used for optimisation and quantitative analysis of the environmental samples in this study using the modified electrode.

4.4.4 Optimisation of the TSA-CP electrode

Cadmium ion determination with the modified electrode was evaluated using square wave anodic stripping voltammetry with the following parameters optimised:

4.4.4.1 percentage of thiosalicylic acid in carbon paste

Voltammetry responses to a 50 µg Cd/L Cd^{2+} solution were studied using electrodes prepared separately with different percentage combinations of thiosalicylic acid (5, 10, 15, 20, 25% w/w), a constant amount of paraffin oil at 24% (w/w), and topped up with

graphite powder. Measurement was conducted in CH_3COONa supporting electrolyte at pH 4.5, with a pre-concentration time of 500 s and 5 Hz sample rate.

4.4.4.2 Supporting electrolyte

The effect of supporting electrolyte on the anodic peak current of the modified electrode was evaluated for Cd^{2+} ion solution concentration of 50 µg Cd/L. The following supporting electrolytes (0.1 mol/L) were used: Hydrochloric acid (HCl), Sodium Chloride (NaCl), Sodium Nitrate ($NaNO_3$) and Sodium Acetate (CH_3COONa). Measurement was conducted using a 15% (w/w) thiosalicylic acid modified electrode, with a pre-concentration time of 500 s and 5 Hz sample rate.

The effect of supporting electrolyte pH on voltammetry responses using the modified electrode was determined in the range of pH 1.5-7.5. The pH of the supporting electrolyte was adjusted by adding HNO_3 (10%) or NaOH (10%) to the supporting electrolyte solution. Measurement was conducted using a 15% (w/w) thiosalicylic acid modified electrode, 0.1 mol/L CH_3COONa supporting electrolyte, with a pre-concentration time of 500 s and 5 Hz sample rate.

4.4.4.3 pre-concentration time

The optimum pre-concentration period was determined by incubating the modified electrode in 50 µg Cd/L Cd^{2+} ion solution for different time periods between 100 and 700 s. The voltammetry response was then measured using a 15% (w/w) thiosalicylic acid modified electrode, 0.1 mol/L CH_3COONa supporting electrolyte, pH 4.5, and 5 Hz sample rate.

4.4.5 Quantitative analysis

4.4.5.1 Linear calibration range of the TSA-CP electrode

A linear calibration range for the modified electrode was determined for Cd^{2+} ion concentrations in the range from 10 to 1000 μg Cd/L. The detection limit was calculated by multiplying the standard deviation of peak current for six determinations of the lowest Cd^{2+} concentration of the linear calibration range by three times (Refera et al., 1998; Fanta and Chandravanshi, 2001).

4.4.5.2 Repeatability and reproducibility of TSA-CP electrode

The repeatability of measurement using the modified electrode was quantified by performing six Cd^{2+} ion determinations for 50, 60 and 80 μg Cd/L Cd^{2+} solutions without renewing the electrode surface. The reproducibility of the modified electrode was performed by conducting six Cd^{2+} ion determinations of 50, 60 and 80 μg Cd/L Cd^{2+} ion solution with renewing the electrode surface (considered as a new electrode each time) after every measurement. The electrode surface was renewed (cleaned) by scraping the electrode on a clean piece of paper to obtain a new electrode surface at each time.

4.4.6 Cation interference ions on Cd^{2+} determination by TSA-CP electrode

Environmental samples may consist of other cations. The potential interfering effect of these cations on the performance of the modified electrode was studied by mixing the Cd^{2+} solution (50 μg Cd/L) with specific interfering cations (Cu^{2+}, Zn^{2+}, Mn^{2+}, Ni^{2+}, Pb^{2+}, Fe^{2+}, Mg^{2+}, Ca^{2+}, Al^{3+} and K^{+}) at a 1: 2 molar ratio (Cd: other metal cation).

4.4.7 The selectivity of TSA-CP electrode towards free Cd^{2+} ions

Ethylenediaminetetraacetate (EDTA) is a hexadentate ligand which forms stable complexes with free Cd^{2+} and reduces the free Cd^{2+} ion concentration in solution. The selectivity of the modified electrode towards free Cd^{2+} ions was determined by measuring the voltammetry responses of the modified electrode for a mixture of Cd^{2+} ion solution (100 µg Cd/L) and Ethylenediaminetetraacetate (EDTA) at 1:1 and 1:2 (Cd: EDTA) molar ratio.

4.4.8 Application of TSA-CP electrode to the analysis Environmental sample

4.4.8.1 Water sample analysis

Industrial wastewater, tap water, and farm drainage water were collected and analysed with the modified electrode to calculate the recovery of Cd^{2+} ions in environmental samples. Samples were separately spiked with 40 and 80 µg Cd/L Cd^{2+} prepared in CH_3COONa (0.1 mol/L). The recovery percentages were tested at spiking ratios of 9:1 (40 µg Cd/L solution: water sample) and 7:3 (80 µg Cd/L solution: water sample) to make the final concentration 36 and 56 µg Cd/L, respectively. A 25 mL aliquot was transferred into a glass cell for pre-concentration and subsequent voltammetric measurement using the modified electrode.

4.4.8.2 Free Cd^{2+} ions in soil solution

Two soil samples (soil A and soil B), each with a different total Cd concentration were collected from a previous pot trial (Chapter 3), were used to test the potential of the modified electrode to quantify the free Cd^{2+} ion concentration in soil solution and to calculate the relative standard deviation (RSD) between measurements. Two replicates

from each soil sample were collected and Cd was extracted from 5 g of soil in 30 mL of 0.1 mol/L CH_3COONa by shaking for 2 hr using an end-over-end shaker. The extracted solutions were filtered and diluted in a 1:1 (v/v) ratio with 0.1 mol/L CH_3COONa solution. A calibration curve for the modified electrode was prepared using five Cd^{2+} ion concentrations (20, 40, 60, 80, 100 μg Cd/L) and this curve was used to quantify the Cd^{2+} ion concentration of both soil samples.

4.4.9 Statistical analysis

Statistical analysis was conducted with Minitab 18 and OriginPro 9 (OriginLab, USA) statistical software. The effect of different levels of thiosalicylic acid, supporting electrolyte pH and pre-concentration time on anodic current peak signal was statistically analysed using a one-way ANOVA test. Where a significant ($P<0.05$) main effect was detected, the difference between treatment means was tested using a Tukey HSD posthoc test.

4.5 Results and discussion

4.5.1 FTIR characterisation of thiosalicylic acid

The FTIR spectrum of thiosalicylic acid from 4000 to 500 cm^{-1} showed two characteristic peaks at 1685 and 2565 cm^{-1} which can be attributed to the -COOH and -SH groups, respectively (Figure 4.1) (Zhou et al., 2011; Yin et al., 2017). The broad peak at 3246 cm^{-1} can be attributed to -OH groups of surface adsorbed water molecules or the OH group in the thiosalicylic acid (Wehr-Candler and Henderson, 2016).

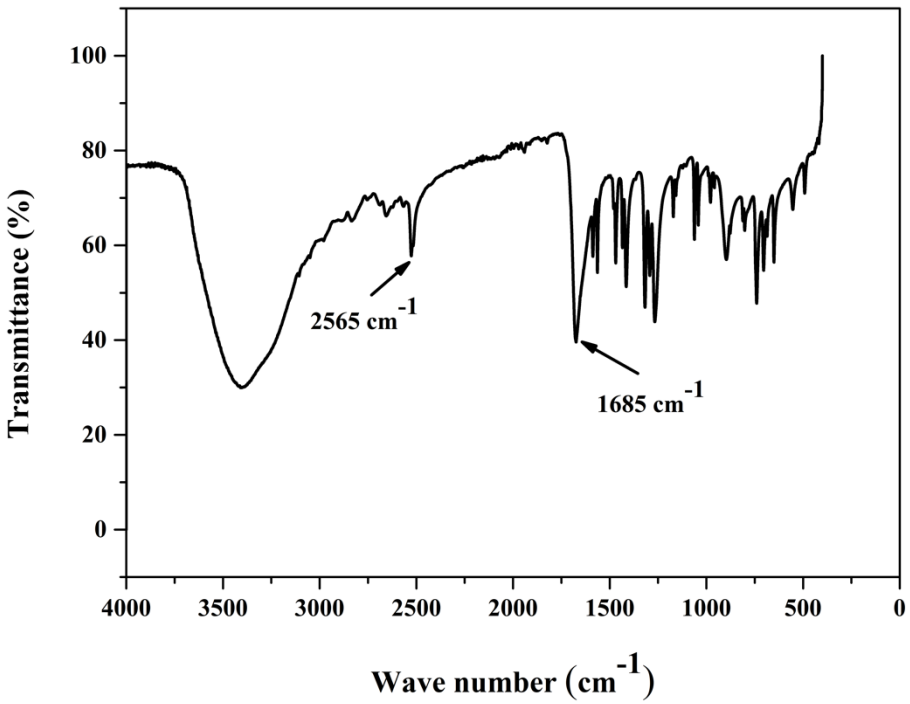


Figure 4.1. FTIR spectrum of thiosalicylic acid from 4000 to 500 cm^{-1} to identify the specific functional groups which can bind with Cd^{2+} ions.

4.5.2 Preliminary voltammetry for Cd^{2+} on TSA-CP electrode

A single high intensity anodic current peak for Cd^{2+} ions was observed for the modified electrode at around -0.8 V potential (Figure 4.2) and can be attributed to Cd accumulation on the modified electrode surface through a mechanism of complex formation between Cd^{2+} and thiosalicylic acid (Gismera et al., 2006). Wehr-Candler and Henderson (2016) reported that the thiol (SH^-) and carboxyl (COO^-) functional groups in thiosalicylic acid can bind with metal ions through four binding modes (i) S atoms coordinate leaving the carboxylic acid free (ii) carboxylate anion binds in a monodentate fashion together with sulphur coordination (iii) both S and O atoms coordinate and (iv) carboxylate anion binds in a bidentate manner with or without S coordination.

The results of the preliminary study confirmed that thiosalicylic acid can be used as a modifier of carbon paste to enable the determination of Cd^{2+} ions in solutions using square wave anodic stripping voltammetry. In subsequent work, optimisation and further development of the modified electrode were carried out to improve the voltammetry response of the modified electrode towards Cd^{2+} ions.

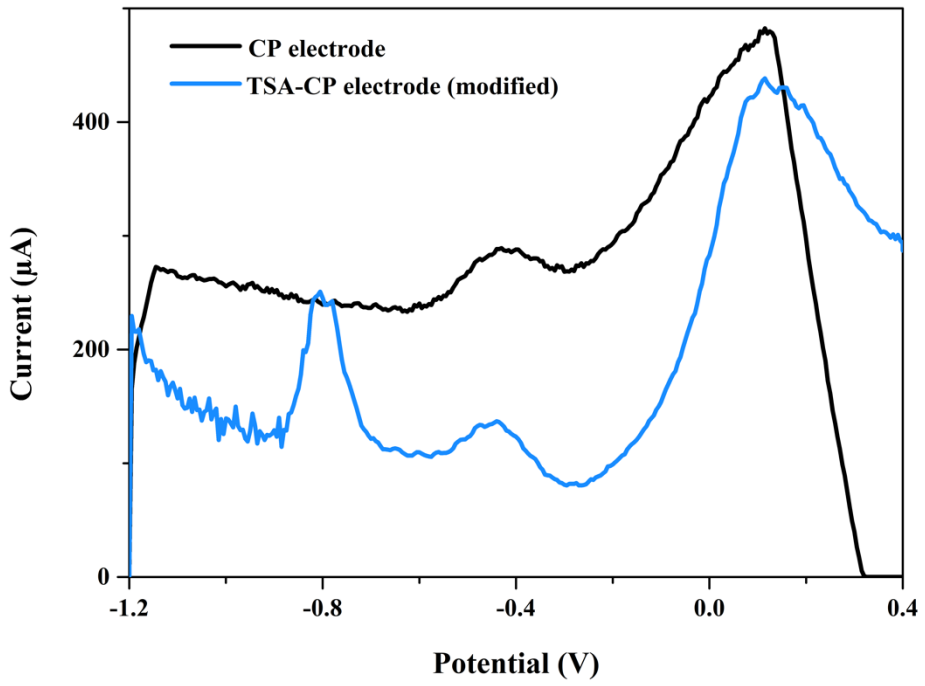


Figure 4.2. Square wave anodic stripping voltammograms of TSA-CP electrode and CP electrode obtained for 50 µg Cd/L Cd^{2+} ion solution. Experimental conditions: 0.1 mol/L CH_3COONa supporting electrolyte (pH 4.5) pre-concentration time; 600 s and sample rate; 5Hz.

4.5.3 Optimisation of the TSA-CP electrode

The optimisation of the modified electrode was quantified through maximising the current peak height at -0.8V and resolving the current signal at -0.8V.

4.5.3.1 Carbon paste composition

Anodic peak current showed a nominal variation across the thiosalicylic acid electrode composition. There was a significant decrease by around 21% in current peak height for the highest percentage of thiosalicylic acid relative to a composition percentage of 15% (w/w) (Figure 4.3).

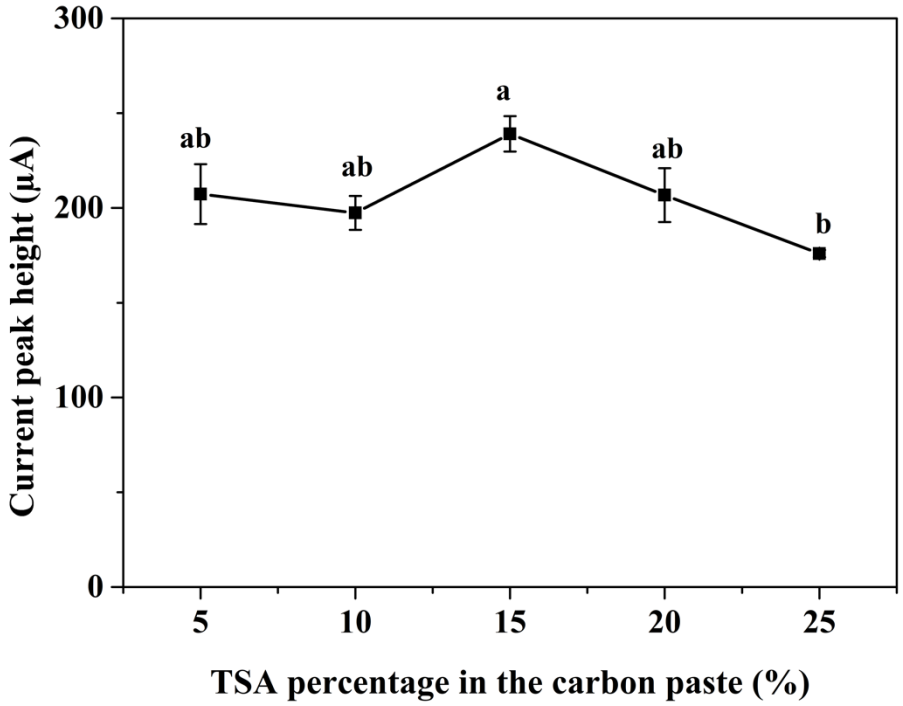


Figure 4.3. Square wave anodic stripping peak current as a function of the amount of thiosalicylic acid in the carbon paste electrode. Experimental conditions: 50 µg Cd/L Cd^{2+} ion solution; 0.1 mol/L CH_3COONa supporting electrolyte (pH 4.5) pre-concentration time; 500s and sample rate; 5 Hz.

Even though the peak current showed nominal variation across the thiosalicylic acid range, the modified electrode recorded a maximum peak current at 15% (w/w). Variations in peak current as a function of thiosalicylic acid in the paste may be due to the rate of Cd^{2+} complex formation with thiosalicylic acid modifier in the electrode surface which

reached a maximum at 15% (w/w) thiosalicylic acid. A reduction of conductive area at the electrode surface may be the reason for the significant ($P<0.05$) decrease of peak current at 25% compared to 15% (w/w) thiosalicylic acid. Studies have reported that other modifiers such as diacetyldioxim and silica used to develop modified carbon electrodes have shown a similar pattern for the current response to Cd^{2+} ions (Hu et al., 2003; Shams and Torabi, 2006). Based on the results, the present study adopted a modified electrode with a composition of 15% (w/w) thiosalicylic acid, 61% (w/w) graphite powder and 24% (w/w) paraffin oil as the optimal combination for subsequent free Cd^{2+} ion measurement. For the current study, 15% (w/w) thiosalicylic acid percentage was selected as the carbon paste thiosalicylic acid composition for ongoing measurement.

4.5.3.2 The type and the pH of the supporting electrolyte

A strong well defined anodic peak current signal (with less background noise) for the modified electrode was obtained using 0.1 mol/L CH_3COONa. A well-defined anodic peak current signal was not recorded for the other electrolytes ($NaNO_3$, NaOH and HCl) (Figure 4.4).

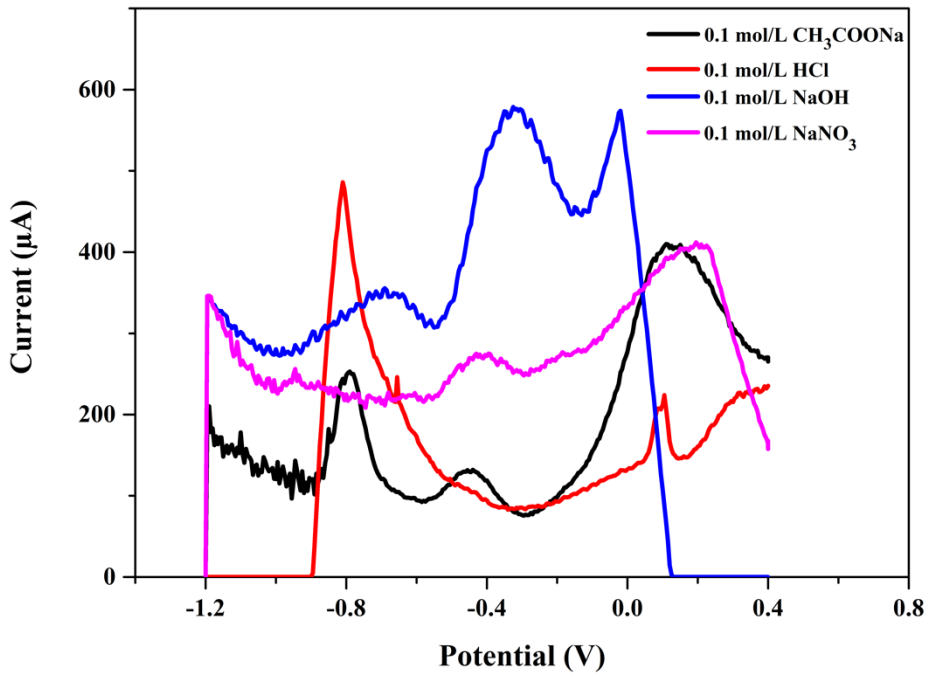


Figure 4.4. Square wave anodic stripping voltammograms as a function of the supporting electrolyte. Experimental conditions: 50 µg Cd/L Cd^{2+} ion solution; preconcentration time; 500s and sample rate; 5 Hz.

The pH of the electrolyte is an important factor for the pre-concentration step due to the effect of pH on thiosalicylic acid Cd^{2+} complexation equilibrium. There was no current observed at pH 1.5 with the peak current significantly increasing ($P<0.05$) as the pH increased from pH 2.5 to 4.5. Maximum peak current was recorded at pH 4.5. Peak current decreased to zero as pH increased from 4.5 to 7.5 (Figure 4.5).

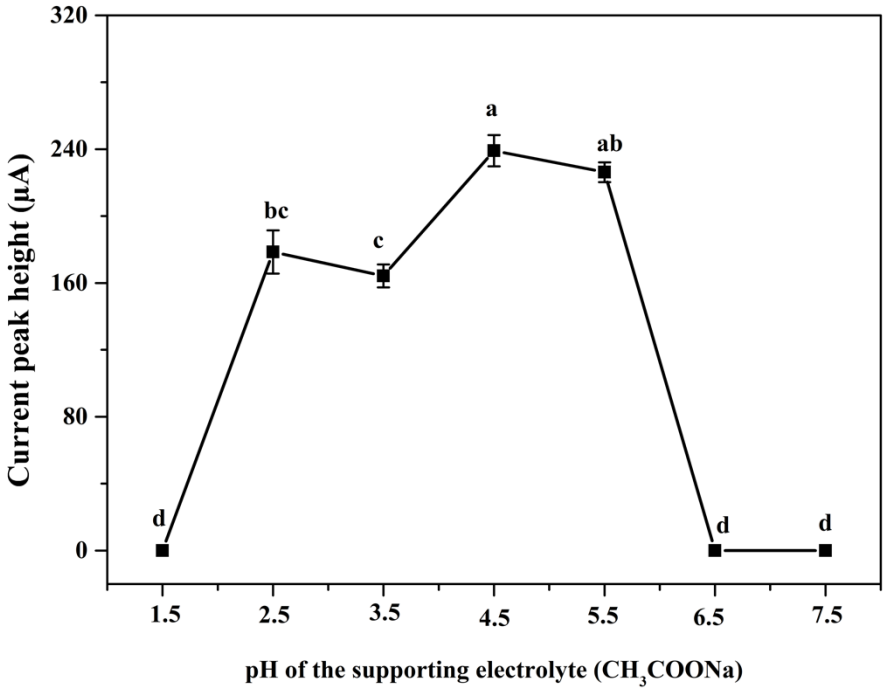


Figure 4.5. Square wave anodic stripping peak current as a function of the pH of the CH_3COONa supporting electrolyte. Experimental conditions; 50 µg Cd/L Cd^{2+} ion solution in 0.1 mol/L CH_3COONa; pre-concentration time; 500s and sample rate; 5 Hz.

The low current signal under highly acidic pH (pH=1.5) may be due to protonation of the ligand whereas precipitation of Cd^{2+} ions in solution (formation of cadmium hydroxide) may explain decreasing current signal electrode at higher pH (pH>4.5) (Abbastabar-Ahangar et al., 2009). Thiosalicylic acid is a diprotic acid with pKa values of 4.92 and 9.96 for the first and second proton dissociations, respectively. Thiosalicylic acid is therefore poorly soluble at lower pH, and readily soluble at higher pH (Wehr-Candler and Henderson, 2016). Rowland et al. (2011) observed 50% oxidation of thiosalicylic acid to the corresponding disulphide ($HOOCC_6H_4SSC_6H_4COOH$) as the pH of the electrolyte increased to 6. Thiosalicylic acid in the carbon paste may, therefore, have limited availability to complex with Cd^{2+} ions at higher pH levels. Available literature reports that other modifiers such as diacetyldioxim and nitro benzoyl diphenyl

methylenphosphorane (N-BDMP) in modified carbon paste electrodes also show a similar pH dependency on the measurement of Cd^{2+} ions in solution (Hu et al., 2003; Afkhami et al., 2012). For the current study, 0.1 mol/L CH_3COONa at pH 4.5 was selected as the supporting electrolyte for ongoing measurement.

4.5.3.3 Pre-concentration time

There was a significant ($P<0.05$) increase in the anodic peak current up to 500 s of pre-concentration time with no further significant change to 700 s, indicating a saturation or equilibrium surface coverage of the electrode surface at 500 s (Figure 4.6). Five hundred seconds was therefore selected as the optimal pre-concentration time for measuring free Cd^{2+} ion using the modified electrode. Fanta and Chandravanshi (2001) suggested that a pre-concentration time greater than 120 s should be used to optimise the detection limit for low Cd^{2+} ions in solution. For the current study, 500 s was selected as the pre concentration-time for ongoing measurement.

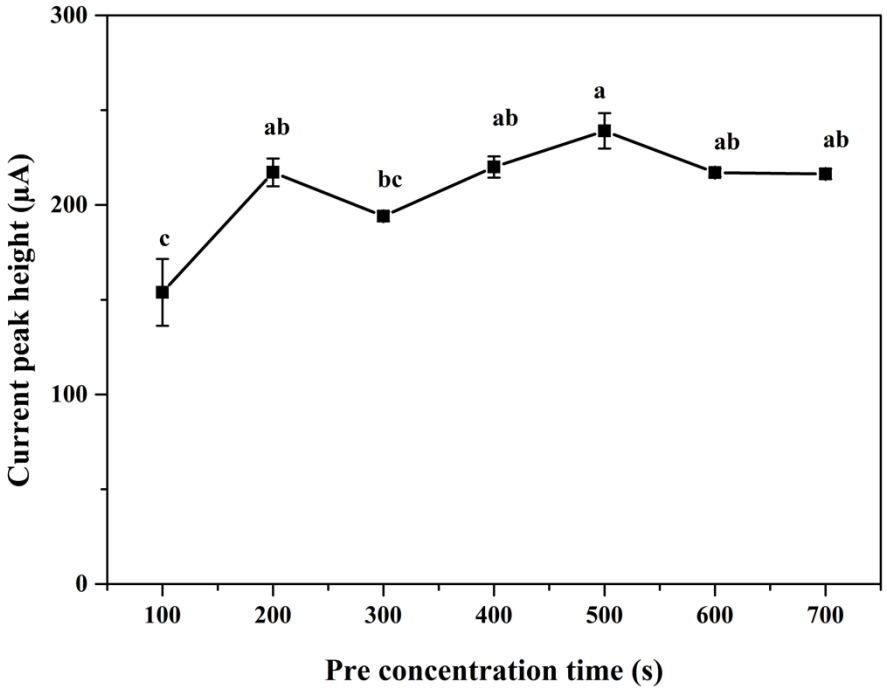


Figure 4.6. Square wave anodic stripping peak current as a function of the pre-concentration time. Experimental conditions; 50 µg Cd/L Cd^{2+} ion solution 0.1 mol/L CH_3COONa supporting electrolyte (pH 4.5) and sample rate; 5 Hz.

4.5.3.4 TSA-CP electrode optimum experimental conditions for Cd^{2+} ion detection

Table 4.1 presents a summary of the optimised values of electrode composition, type and pH of the supporting electrolyte and pre-concentration for the modified electrode fabricated in the current study for free Cd^{2+} ion detection in solution.

Table 4.1. Optimized parameters for Cd^{2+} ion detection using modified electrode.

Parameter	**Optimised value**
Electrode composition	15% (w/w) Thiosalicylic acid, 24% (w/w) paraffin oil, 61% (w/w) graphite powder
Supporting electrolyte	0.1 mol/L CH_3COONa buffer
the pH of the supporting electrolyte	pH 4.5
Pre-concentration time	500 s
Sample rate	5 Hz
Pulse amplitude	0.05 V

4.5.4 Performance of the optimised electrode

4.5.4.1 Calibration linear range and detection limit of the TSA-CP electrode

The linear range of a calibration curve is the range of concentrations where the signals are directly proportional to the concentration of the analyte in the sample. Understanding the linear range is important for the accurate detection of samples and the interpretation of results. The linear range of the modified electrode was observed, for a concentration from 20 to 100 µg Cd/L (Figure 4.7). The detection limit, defined as three times the standard deviation of peak current for six determinations of the lowest Cd^{2+} concentration of the linear calibration range (20 µg Cd/L) was calculated as 11 µg Cd/L for the modified electrode.

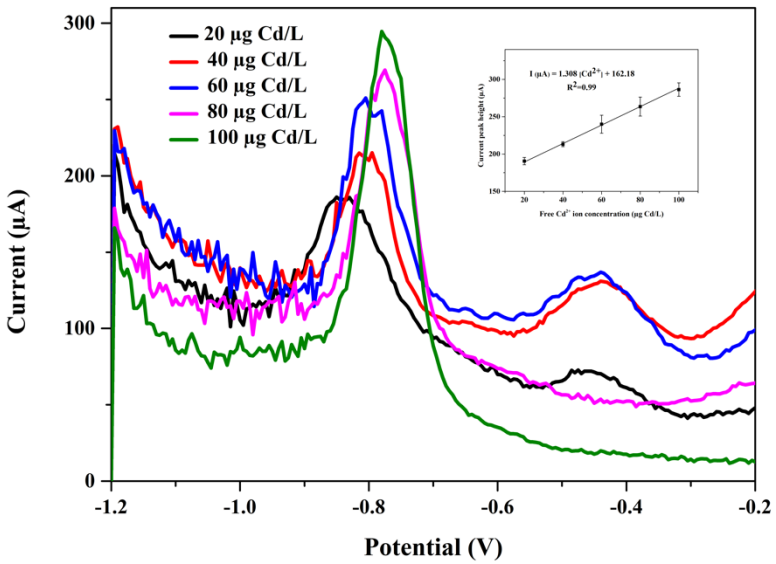


Figure 4.7. Square wave anodic stripping voltammograms obtained using the TSA-CP electrode for different Cd^{2+} ion concentrations; Experimental conditions: 0.1 mol/L CH_3COONa (pH 4.5) pre-concentration time; 500s and sample rate; 5 Hz.

4.5.4.2 Repeatability and reproducibility of the TSA-CP electrode

The repeatability of Cd measurement, assessed by performing six determinations with the same standard concentration of three different Cd^{2+} ions concentrations (50, 60 and 80 µg Cd/L) without renewing the electrode surface, resulted in an RSD of 6.8%, 7.5% and 13.9%, respectively. The reproducibility of the electrode response, assessed by using six different modified electrodes in the presence of 50, 60 and 80 µg Cd/L Cd^{2+} ion solutions, showed an RSD of 2.1%, 3.5% and 1.5%, respectively. To perform reproducibility measurements, a new electrode was made for each measurement by cleaning (scraping) the previous electrode surface against a clean paper to remove surface residues from the previous analysis. The RSD for electrode response measured without renewing the electrode surface was higher than for the electrode response measured with renewing the electrode surface. These results support renewing the electrode surface by scraping against a clean paper prior to each Cd^{2+} ion measurement performed by the modified electrode.

4.5.5 Interference studies

High selectivity is an important characteristic of any carbon paste electrode developed to measure trace metal concentrations in solution. In the context of the current study, other cations in solution could potentially compete with Cd^{2+} ions for thiosalicylic acid binding sites on the electrode surface and interfere with peak signal (Fanta and Chandravanshi, 2001). Possible interferences by Ni^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, Pb^{2+}, Cu^{2+}, Mg^{2+}, Ca^{2+}, K^{+} and Al^{3+} with the anodic stripping voltammetry of Cd^{2+} ions were therefore investigated. At a

free Cd^{2+} concentration of 50 µg Cd/L, the metal interference of each metal ion was less than 21% except for Pb^{2+}, Cu^{2+} and Fe^{2+} (Table 4.2).

Table 4.2. The interference effect of solution cations at a concentration of 100 µg Cd/L concentration on the anodic peak current for a Cd^{2+} concentration of 50 µg Cd/L quantified using the modified electrode.

Cation	**Ni^{2+}**	**Zn^{2+}**	**Mn^{2+}**	**Fe^{2+}**	**Pb^{2+}**	**Cu^{2+}**	**Mg^{2+}**	**Ca^{2+}**	**K^{+}**	**Al^{3+}**
Change in the peak current (%)	-20	-8.3	18.8	38	65	55	-8.6	-21.3	-7.4	-2.7

Note: Change in the peak current refers to the percentage variation of the square wave anodic peak current corresponding to the 50 µg Cd/L Cd^{2+} ion in the presence of other cations at 100 µg Cd/L concentration.

The variable interference shown by the tested metal cations may be due to variability in the binding affinity of the cations with thiosalicylic acid (Perrin, 1958). Gismera et al., 2003) and Gismera et al. (2006) reported that thiosalicylic acid shows a great affinity for soft trace metal ions such as Pb^{2+} and Cu^{2+} and reported the detection limit of thiosalicylic acid-modified electrodes for Pb^{2+} and Cu^{2+} ions as 828×10^{-14} µg Pb/L (4×10^{-8} mol Pb/L) and $63.5 \times 10^{-12.3}$ µg Cu/L ($1 \times 10^{-6.3}$ mol Cu/L), respectively which are lower than the detection limit for Cd^{2+} in the current study. Perrin (1958) reported that the Cu-thiosalicylic acid (pK1-10.60) and Fe-thiosalicylic acid (pK1-16.35) complexes have higher stability constants than Cd-thiosalicylic acid (pK1-5.55) complexes. Fanta and Chandravanshi (2001) observed Cu^{2+} and Pb^{2+} ions can significantly interfere to suppress the Cd^{2+} signal of a N-P-Chlorophenyl-cinnamohydroxamic acid (CPCHA) modified electrode, as these cations can form complexes with CPCHA and prevent the accumulation of Cd at the electrode surface. Chamjangali et al. (2015) reported that a bismuth film/crown ether/Nafion modified screen-printed carbon electrode showed a 1:1 tolerance limit for Cu^{2+} ions when detecting Cd^{2+} ions in solutions due to the deposition of intermetallic Cu-Cd compounds on the electrode surface. Evidence from literature and

the current study suggest that thiosalicylic acid used for the modified electrode has a greater affinity towards Cu^{2+} and Pb^{2+} ions and substantiates the potential for high interference of these ions on the detection of Cd^{2+} in solution. However, studies have reported that interference of Cu^{2+} ions could be easily eliminated by adding ferrocyanides into the electrolyte solution to produce an insoluble and stable copper ferrocyanide complex (Hao et al., 2016; Zhao et al., 2016).

4.5.6 The selectivity of the TSA-CP electrode towards free Cd^{2+} ions

The purpose of using an ion-specific electrode is to quantify the free ion concentration in the solution. Verification of the performance of any new electrode must therefore analyze the performance of the electrode in discriminating between free and complexed ions. Ethylenediaminetetraacetate forms stable complexes with free metal ions by forming a ring structure with the metal ion via nitrogen and oxygen atoms in the EDTA molecule and can be used to quantify the selectivity of an electrode to free ions (Xie, 2009). Voltammograms of Cd^{2+} ion peak current variation in the absence of EDTA and at a Cd: EDTA molar ratio of 1:1 and 1:2 is shown in Figure 4.8. The peak current corresponding to Cd^{2+} reduced as the EDTA concentration in the solution increased and was not detected at a Cd^{2+}: EDTA molar ratio of 1:2.

Tanaka et al. (1956) also reported a reduction in peak current for free Cd^{2+} ions in a 1:1 molar ratio of Cd: EDTA using a mercury hanging drop electrode as the indicator electrode in pH 4.2 acetate buffer. The reduction of Cd^{2+} current peak may be due to the complexation of free Cd^{2+} ions with EDTA in solution. This infers that the modified electrode has the ability to distinguish between total Cd and free Cd^{2+} in the solution and measure only the free Cd^{2+} ions and is evidence of a major advantage of the modified

electrode compared to other techniques such as AAS, ICP-AES and MP-AES (Baffi et al., 2002).

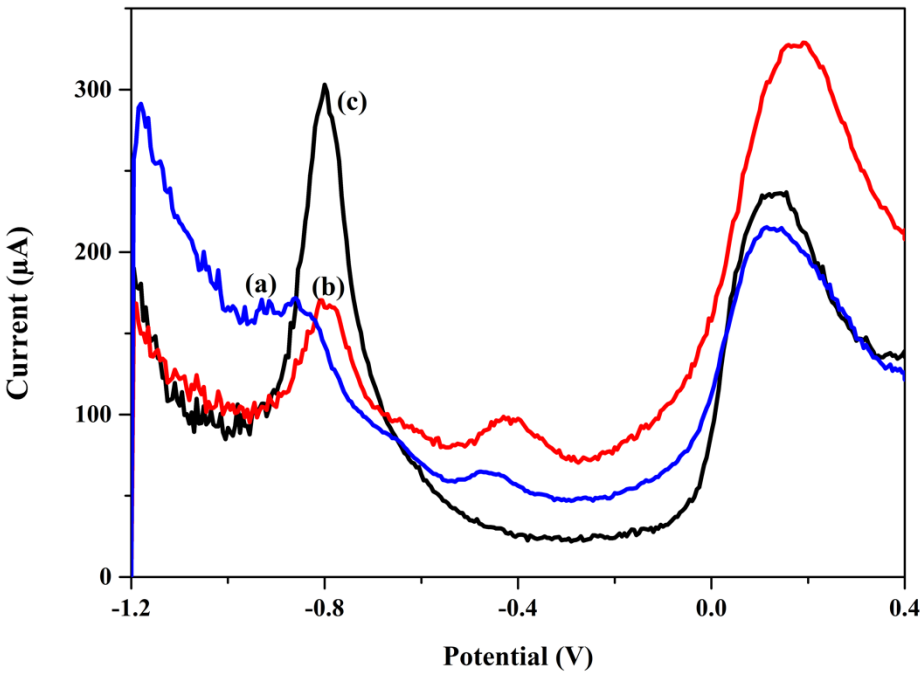


Figure 4.8. Square wave anodic stripping voltammograms for Cd^{2+} ions as a function of increasing EDTA concentrations at (a) 1:2 Cd: EDTA (b) 1:1 Cd: EDTA (c) no EDTA. Experimental conditions; 100 µg Cd/L Cd^{2+} ion concentration in 0.1 mol/L CH_3COONa (pH 4.5), preconcentration time; 500 s and sample rate; 5 Hz.

4.5.7 Application to environmental samples

To test the applicability of the optimised modified electrode to environmental samples, the free Cd^{2+} ion concentration was analysed in a range of water and soil solution matrices. For water analysis, water samples from three different sources; tap water, farm drainage water and wastewater were selected to progressively investigate the effect of interference ions on the determination of free Cd^{2+} ions. Soil solution was analysed to

investigate the precision of Cd^{2+} ion detection using the modified electrode for 'real life' samples that might be analysed in future research.

4.5.7.1 Water sample analysis

To test the applicability of the modified electrode to water samples; drainage water, wastewater and tap water were spiked with Cd to achieve two concentrations of Cd in solution. The analytical recovery of added free Cd ions was 103%, 78% and 103%, for a spiked concentration of 40 µg Cd/L changed to 84%, 82% and 91%, for a spiked concentration of 80 µg Cd/L (Table 4.3). Analytical recovery at both spiked Cd concentrations in wastewater was lower than for drainage and tap water. Cation analysis of all water samples showed that wastewater contained a higher concentration of interfering ions when compared to drainage and tap water (Appendix 3). Therefore, it can be suggested that lower Cd^{2+} recovery percentages for the wastewater samples were due to the high concentration of interfering metal ions in the wastewater samples.

Table 4.3. Analytical recovery of Cd^{2+} ions from water samples using the TSA-CP electrode.

Water Sample	**Added [Cd^{2+}] (µg Cd/L)**	**Measured [Cd^{2+}] (µg Cd/L)**	**Analytical recovery (%)**
Drainage water	36	37	103
	56	47	84
Wastewater	36	28	78
	56	46	82
Tap water	36	37	103
	56	51	91

Data are means±standard errors of three replicates (n=3).

4.5.7.2 Soil solution analysis

To accurately assess the performance of any newly developed analytical technique, it is important to evaluate the precision of results across different environmental matrices. To

investigate the precision of the modified electrode, the free Cd^{2+} ion concentration in soil solution from two soil samples was analysed and the RSD between soil replicates was calculated. The RSD for extractable Cd^{2+} ion concentration between two soil samples of soil A, collected from two different experimental pots with the same Cd concentration was, 0.7%, and that for soil B was 1.6% (Table 4.4). The low RSD between two replicates for each soil sample (soil A and soil B) suggest that the modified electrode shows a high precision in Cd^{2+} ion determination in environmental samples (soil solutions). Comparison of the results from the electrode to the total Cd concentration in soil solution (quantified using GFAAS) shows that 94% and 89% of total Cd was in the form of free Cd^{2+} ion for the two replicates for soil A, whereas for soil B free Cd^{2+} ion concentration was 96% and 89% of total Cd.

Table 4.4. Determination of free Cd^{2+} ion concentration using the TSA-CP electrode and the total Cd^{2+} ion concentration using GFAAS of the soil solution.

Sample name	**Measured free Cd^{2+} ion concentration (µg Cd/L)**	**RSD (%) of free Cd^{2+} concentration of soil between two pots of each soil type**	**Measured total Cd^{2+} ion concentration (µg Cd/L)**
Soil A (Pot 1)	191.1±4.4		202.3±13.4
Soil A (Pot 2)	192.2±5.6	0.7	214.7±24.7
Soil B (Pot 1)	174.2±1.1		180.9±38.2
Soil B (Pot 2)	169.7±2.2	1.6	188.8±58.4

Data are means±standard errors of three replicates (n=3). RSD (%) was calculated between the Cd^{2+} ion concentration of soil collected from two pots of each soil sample type.

4.5.8 Validation of TSA-CP electrode

Understanding the mechanisms of Cd uptake and translocation by plant species requires knowledge of the free Cd^{2+} ion concentration in xylem saps. However, the determination of low concentrations of free Cd^{2+} ions in the low volume of xylem saps poses an analytical challenge. The modified electrode developed and verified in the current work

has been shown to distinguish between total Cd and free Cd^{2+} in the solution and measure only the free Cd^{2+} ions in environmental samples. Furthermore, the electrode exhibited a high precision in the measurement of low Cd^{2+} concentrations in a low volume of environmental matrices. Free Cu^{2+} and Pb^{2+} ions had considerable interference on the detection of free Cd^{2+} ions and there may be minor interference from these metal ions in determining free Cd^{2+} in xylem saps; however, it might not be a significant issue due to the high complexation of free Cu^{2+} and Pb^{2+} with organic anions in xylem saps (Ghnaya et al., 2013). The specific characteristics of the thiosalicylic acid modified electrode described in this work underpin its use as a reliable and promising tool to determine the free Cd^{2+} ion concentration in chicory and plantain to understand the major form of Cd translocation in chicory and plantain saps. Therefore, in the next chapter thiosalicylic acid modified electrode will be used to determine the free Cd^{2+} ion concentration in the chicory and plantain xylem sap to understand the Cd translocation. Knowledge of the free Cd^{2+} ion concentration in xylem saps will increase understanding of the Cd translocation mechanisms in chicory and plantain.

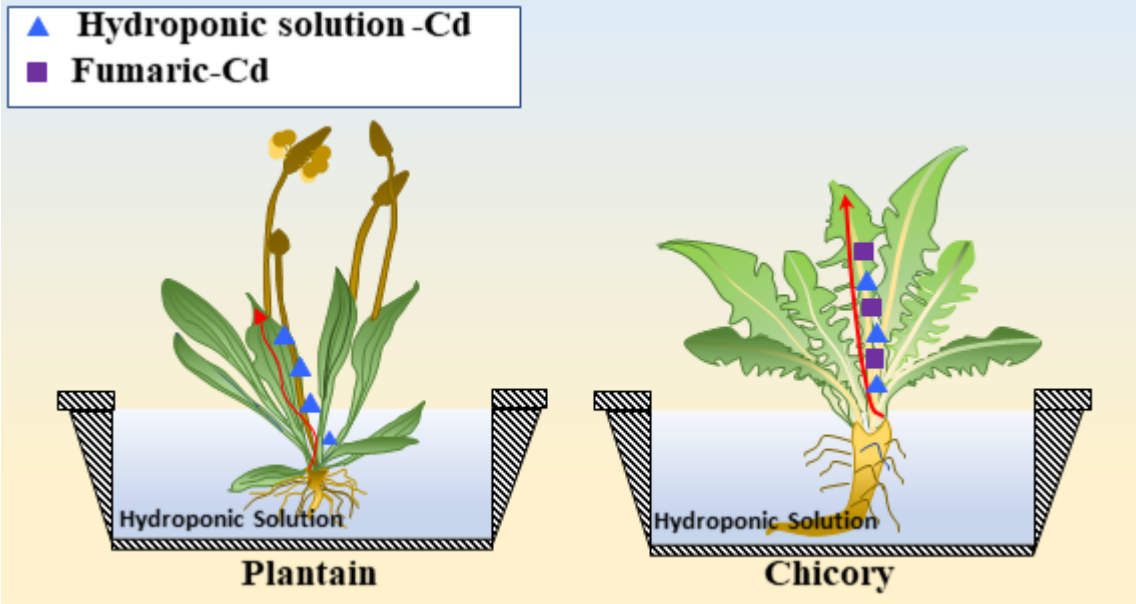
Chapter 5

Influence of cadmium in growth media on organic acid production in the xylem sap of chicory and plantain

Part of the results of this chapter was presented in the proceedings of the Farmed Landscape Research Workshop 2020 Citation:

Ubeynarayana, N., Jeyakumar, P., Bishop, P., Calvelo Pereira, R. and Anderson, C., 2020. Complexation of cadmium with organic acids in xylem fluid of chicory and plantain. In: Nutrient Management in Farmed Landscapes. http://flrc.massey.ac.nz/publications.html. Occasional Report No. 33. Farmed Landscape Research Centre, Massey University.

5.1 Graphical Abstract



5.2 Abstract

The effect of Cd on xylem sap LMWOA production and Cd translocation was examined in chicory and plantain plants grown in hydroponic solution amended with six Cd levels: 0 (Control), 0.01, 0.1, 0.5, 2.5 and 5 mg Cd/L for 90 days. Both chicory and plantain showed variable production of oxalic, fumaric, citric, malic and acetic acids as a function of Cd treatment in the xylem sap. There was a significantly higher shoot Cd concentration in chicory than plantain in all Cd treatments, except for the control and 0.01 mg Cd/L treatments. The significantly higher shoot Cd concentration in chicory can be explained in terms of variations of LMWOA production in both plants. LMWOA concentrations in chicory xylem sap showed a significant ($P<0.05$) and positive correlation with the shoot and xylem sap Cd concentration. However, there was no significant ($P>0.05$) correlations observed between LMWOA concentrations with shoot and xylem sap Cd concentration for plantain. Although all LMWOAs produced in chicory xylem sap significantly correlated with shoot Cd concentration, stepwise regression analysis showed that the primary cause for higher shoot Cd concentration in chicory relative to plantain is fumaric acid production which may bind with chicory in xylem sap and translocate the metal towards shoots. To evaluate the applicability of the regression model to plants grown in field conditions a soil experiment was conducted by growing chicory and plantain in Allophanic, Gley and Recent soils, separately. The derived regression model was used to predict the shoot Cd concentration of chicory grown in three types of New Zealand pastoral soil. There was a significant and positive correlation ($R=0.925$, $P<0.001$) between the predicted and actual shoot Cd concentration of chicory grown in each soil type.

5.3 Introduction

Xylem-mediated root-to-shoot translocation is the main process accounting for Cd accumulation in the aerial parts of plants (Uraguchi et al., 2009). Analysis of metal speciation in xylem sap via X-ray fluorescence (Cheng et al., 2016) and X-ray absorption spectroscopy (Lu et al., 2013) has previously shown that metal translocation to shoots occurs through complexation with LMWOAs. Elevated concentrations of trace metals including Cd in the soil can activate various enzymes in the TCA cycle of plants which are responsible for the production of LMWOAs inside plant cells (Tatár et al., 1998; Mnasri et al., 2015). The complexation of these LMWOAs with metal ions can play an important role in metal translocation. For example, Senden and Wolterbeek (1990) reported that metal-LMWOA complexes, such as Cd-citrate, in tomato plants, could transport Cd efficiently from root to shoot tissues via the xylem sap. Li et al. (2019b) reported that the citric acid concentration in rice (variety Luhui 17) xylem sap increased from 0.51 to 0.60 mg/L when the Cd concentration in hydroponic media increased from 0 to 2 mg Cd/L. In another study, the concentration of citric and tartaric acids in rice (Lu.527-8) xylem sap was positively correlated (R=0.82 and R=0.97, respectively) with total xylem sap Cd concentration, when the soil Cd concentration increased from control to 10 mg Cd/kg soil (Fu et al., 2019).

The translocation of Cd as an LMWOA complex may be beneficial to plants. Many researchers have reported that metal detoxification inside a plant can happen as complexation reduces the free ionic form of metals in the xylem sap (Pence et al., 2000). Pence et al. (2000) reported that the pennycress synthesizes more organic acids when it is subjected to high Cd^{2+} concentrations in hydroponic solution to avoid the toxicity of free Cd^{2+} ions in xylem sap. For an increase in Cd levels from 0 to 11.2 mg Cd/L, Dresler et al. (2014) reported an increase in malate and citrate production by 142% and 242%,

respectively, in mature leaves of maize with a corresponding increase in leaf Cd concentration from 0-250 mg Cd/kg DW without any indication of plant stress. They proposed that the production of malate and citrate in the TCA cycle is involved with both Cd detoxification and translocation in maize.

Literature provides evidence that Cd enhances the specific production of LMWOAs in xylem sap, and that the involvement of LMWOAs in xylem sap Cd translocation varies between plant species and cultivars (Montargès-Pelletier et al., 2008; Li et al., 2019b). The influence of LMWOAs on xylem sap Cd translocation mechanisms in forage species such as chicory and plantain remain under-studied and such information is useful for alleviating the risk of Cd transfer into the food chain. Advances in understanding the mechanism for xylem sap Cd translocation in chicory and plantain, therefore, requires knowledge of how soil solution Cd concentration can induce LMWOA variation in xylem sap. The specific objectives for this study were: (a) to determine the impact of Cd in hydroponic solution on xylem sap LMWOA production and investigate the influence of Cd-LMWOA complex on Cd translocation in chicory and plantain; and (b) to develop a statistical model based on hydroponic data that can be used to predict the shoot Cd concentration in chicory and plantain when grown in different soil types of New Zealand.

5.4 Materials and methods

The forage species of chicory (*Cichorium intybus* L.) and plantain (*Plantago lanceolata* L.) were simultaneously grown separately in hydroponic solution and soil.

5.4.1 Experiment one: Hydroponic experiment

A hydroponic experiment was set up in the greenhouse at the Massey University Plant Growth Unit with a day/night temperature of 17/20 ℃. A modified Hoagland solution was used as hydroponic media and spiked with a calculated amount of $CdCl_2$ equivalent to six different Cd treatments: 0 (control), 0.01, 0.1, 0.5, 2.5 and 5.0 mg Cd/L. The compositions of the hydroponic solution were; 1003 mg/L (5 mM) $Ca(NO_3)_2 \cdot 2H_2O$, 505.5 mg/L (5 mM) KNO_3, 493.2 mg/L (2 mM) $MgSO_4 \cdot 7H_2O$, 136.2 mg/L (1 mM) KH_2PO_4, 3.6 mg/L (0.1 mM) EDTA-Fe, 2.8 mg/L (47 μM) H_3BO_3, 0.2 mg/L (1 μM) $MnCl_2 \cdot 4H_2O$, 0.2 mg/L (1 μM) $ZnSO_4 \cdot 7H_2O$, 1 μg/L (0.01 μM) H_2MoO_4 and (0.05 mg/L) 0.25 μM $CuSO_4 \cdot 5H_2O$ (Xin et al., 2017a). Prior to the experiment chicory and plantain seeds were germinated on microfiber sponges in green plastic cups for 10 days on a germination bench at 17/20 ℃ (Figure 5.1a). After germination three healthy and uniform seedlings of each plant were transplanted into a container containing hydroponic solution (50 L), which was covered by polyvinyl plates with three smoothly round holes (Figure 5.1b). The experiment was arranged in a completely randomized design with six treatments (6 containers) per plant and three replicated plants per treatment for 90 days. The hydroponic media was renewed every 7 days as well as half an hour before the collection of the xylem sap, and the pH of the solution was adjusted to 5.5-6.0 every day (Liao et al., 2000) by 0.1M HNO_3 acid to prevent significant depletion of nutrients and changes in solution pH[1].

[1] The pH of the hydroponic solution checked every day. The hydroponic solution pH was adjusted to pH 5.5-6.0 on the first day of the experiment and it was constant for first three days. However, it was adjusted to pH range 5.5-6.0 and maintained after third day.

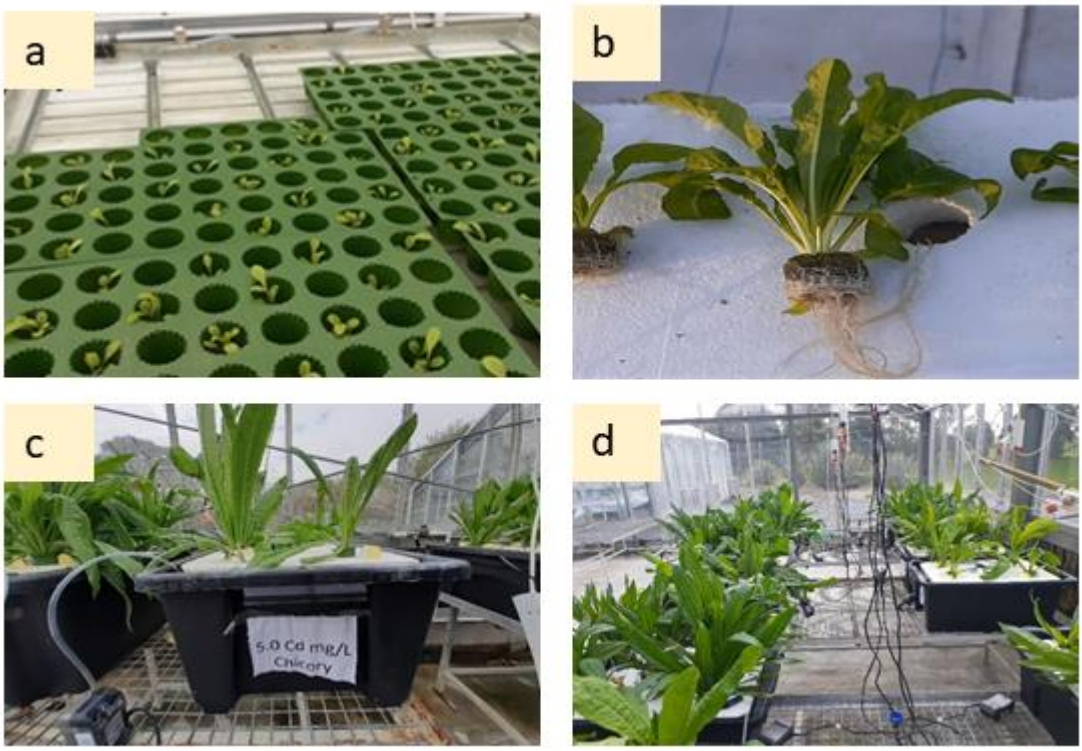


Figure 5.1. Experimental steps of the hydroponic experiment. (a) germination of chicory and plantain seeds on microfiber sponges in green plastic cups (b) growth of chicory plant on microfiber sponge (c) Aeration of the hydroponic containers via aquarium pumps (d) Arrangement of the greenhouse set up.

5.4.2 Experiment two: Pot experiment

A parallel pot experiment was set up in the same greenhouse under the same environmental conditions. Bulk samples of three representatives NZ pastoral soils from the Allophanic, Gley and Recent soil orders (Cryands and Udands, Aquepts, and Dystric Fluventic Eutrudept, respectively in the US Soil Taxonomy Classification; (Hewitt, 2010)) were collected (0-150 mm depth) from three dairy farms located in Waikato (37°41'52.1"S 175°37'26.9"E), Canterbury (43°39'11.6"S 172°28'11.1"E) and Palmerston North (400 22’ 55. 56’’ S 1750 36’ 21. 37’’ E), respectively. These three soil types were selected based on their different soil chemical characteristics. The soils could be classified by their Cd concentration as high (Allophanic-0.94 mg Cd/kg), medium

(Gley-0.74 mg Cd/kg) and low (Recent-0.21 mg Cd/kg) Cd soils. The collected soil was air-dried at 30 ℃ for 5 days, sieved through a <4 mm sieve and 18 pots (2 L) were filled with 1kg of the soil for an experimental set up of 2 plants (chicory and plantain) x 3 soil types x 3 replicates. A bulk sub-soil sample from each soil type was further sieved through a 2 mm stainless steel sieve and stored for soil characterisation (Table 5.1). Prior to the experiment chicory and plantain seeds were germinated on germination paper for 10 days in a germination laboratory at 17/20 ℃. After germination, one viable and healthy seedling of each plant was planted in the middle of the soil-filled pots. The greenhouse experimental setup was arranged in a Complete Randomised Design (CRD) and maintained at a pot-field capacity of 70% for 90 days in a greenhouse where the average day/night temperature ranged between 17 and 20 ℃.

Table 5.1. Chemical properties of Allophanic, Gley and Recent soil used in the study.

Soil type	pH	TOC (g/kg)	OM (g/kg)	Total Cd (mg Cd/kg)
Allophanic	5.3	91.0	182.0	0.97
Gley	5.5	57.0	114.0	0.74
Recent	5.9	27.2	55.4	0.28

5.4.3 Plant harvest and soil sampling

Ninety days after transplanting, plant shoots and roots were removed separately from both pot and hydroponic experiments and xylem sap was collected as described in section 5.4.4. Immediately after harvest roots were dipped in a cold 0.36×10^{-3} mg/L HCl solution to eliminate external Cd adsorbed at the root surface (Ghnaya et al., 2013).

The rhizosphere soil (defined as soil adhered to the plant roots) from the pot experiment was collected by gently scraping the soil from roots by hand (Jeyakumar et al., 2014; Xin et al., 2015). All rhizosphere soil samples were air-dried at room temperature and ground

to pass through a 2 mm stainless steel sieve and stored in sealed plastic bags at room temperature until analysis.

5.4.4 Xylem sap collection

The xylem sap from all plants (from experiment 1 and experiment 2) was collected by the method described by Liao et al. (2000) with modifications. Briefly, the chicory and plantain stems were cut using a stainless-steel razor blade at about 1 cm above the media surface perpendicular to the stem axis. To avoid contamination of the xylem sap with cell sap through the cutting wound, the first drop of exudate was rejected. The xylem sap was collected with a micropipette. The collection time of day can cause variations in xylem sap Cd concentrations due to diurnal variations of plant metabolic activities (Liao et al., 2000). Therefore, xylem sap was collected between 0800-0930 hrs (NZT) to maintain consistency in sap collection. Collected sap samples were immediately passed via a 0.45µm filter and frozen at a -80 ˚C freezer until further analysis.

5.4.5 Plant analysis

5.4.5.1 Plant biomass

Plant shoots and roots were separated at harvest after collecting the xylem sap and dried at 60 ˚C to constant weight. The total dry weight of each plant portion was recorded. Dried shoot and root biomass were ground using a Cyclotech mill and stored for further chemical analysis.

5.4.5.2 xylem sap total Cd concentration

Xylem sap Cd concentration was determined based on the method explained by Nakamura and Akiyama (2008) with modifications. Briefly, 10 µL of xylem sap was digested with 990 µL of 2% HNO_3 acid at room temperature for 1 hr, and the Cd concentration in the digested solution was determined by GFAAS.

5.4.5.3 Plant tissue total Cd concentration

For each shoot and root biomass sample, 0.1 g of dried and ground material was digested with conc HNO_3 (10 mL) and diluted up to 25 mL with de-ionized water. The total Cd concentration in the digested solutions was determined by GFAAS. The shoot to root translocation factor (TF) was calculated as the ratio of shoot Cd concentration to root Cd concentration (Jeyakumar et al., 2010).

5.4.5.4 Free Cd ion concentration in xylem sap

The free Cd^{2+} ion concentration in chicory and plantain xylem sap was determined for the 5 mg Cd/L and 2.5 mg Cd/L hydroponic treatments[2]. Briefly, the collected xylem sap was diluted with 0.1 mol/L sodium acetate (CH_3COONa) solution at 1:20 (v/v) ratio and the free Cd^{2+} ion concentration was measured using the thiosalicylic acid modified carbon paste electrode developed in Chapter 4. A calibration curve was prepared using five Cd^{2+} ion concentrations prepared in 0.1 mol/L CH_3COONa (20, 40, 60, 80, 100 µg Cd/L) and the curve was used to calculate the Cd^{2+} ion concentration in plant samples.

[2] The plants grown at 5 and 2.5 mg Cd/L treatments were selected to analyse the free Cd^{2+} ion concentration using thiosalicylic acid modified electrode due to higher xylem sap LMWOA and higher xylem Cd^{2+} concentration in plants grown at 5 and 2.5 mg Cd/L treatments.

5.4.5.5 HPLC analysis for LMWOAs in xylem sap

The composition and concentration of LMWOAs in xylem sap were analysed by High-Performance Liquid Chromatography HPLC (Agilent Technologies 1200 Series, Santa Clara, CA, USA) as described by Cawthray (2003) and Nakamura and Akiyama (2008) with modifications. Ten µL xylem sap extracted from plant tissues was diluted with 990 µL of 25 mM KH_2PO_4 (the HPLC mobile phase solution). Each mixture was subsequently filtered through a 0.22 µm filter to remove suspended material prior to injection into the HPLC. Separation was conducted on a 250 × 4.6 mm (5 µm particle size) C_{18} reverse-phase column. Each sample solution (100 µL) was injected into the column with a flow rate of 1.0 mL/min at 25°C and UV detection at 210 nm. Potassium dihydrogen phosphate (25 mM) solution was used for isocratic elution. Identification of organic acids was performed by comparing retention times in xylem sap samples with those retention times obtained by analysing a standard mixture including six common LMWOA: (i.e. acetic, citric, fumaric, malic, oxalic, and tartaric), which are usually present in xylem exudates (Fu et al., 2019).

5.4.6 Soil Analysis

5.4.6.1 Total and bioavailable Cd concentration of the rhizosphere soil

One gram of rhizosphere soil from the pot experiment was digested with conc HNO_3 (10 mL) and diluted to 25 mL with de-ionized water. The total Cd concentration in each digested solution was determined by GFAAS. For bioavailable soil Cd concentration, 30 mL $CaCl_2$ (0.05 mol/L, pH 7) was added to 5 g of soil in a centrifuge tube and shaken in an end-over-end shaker for 2 hr at room temperature. The solution was centrifuged at

1068 g for 10 min and filtered through Whatman 42 filter paper. The filtered solution was then analyzed using GFAAS.

5.4.7 Quality control measures

All chemicals used in the experiments were of analytical grade. The limit of detection for Cd for total Cd concentration in this work was 0.002 mg Cd/L. The accuracy of the measurements was assessed by analyzing certified reference materials in parallel with unknown samples. For soil total Cd concentration, NIST SRM 2710a Montana soil I sample, (total Cd 12.3 mg Cd/kg), was used as the certified reference material. The mean Cd concentration of the NIST SRM 2710a was obtained as 12.0±1 mg Cd/kg, which is 90-106% of the expected value. For plant total tissue Cd analysis, NIST 1573a (National institute of standards and technology, tomato leaves-1.52 mg Cd/kg) was used as certified reference material and found to be within 93-105% of the expected mean value.

5.4.8 Statistical analysis

Statistical analysis was conducted with Minitab 18 and OriginPro 9 (OriginLab, USA) statistical software. The effect of Cd treatments on different plant and soil variables was statistically analysed using a one-way ANOVA test; if a significant ($P<0.05$) main effect was detected, the difference between treatment means was tested using a Tukey HSD posthoc test. The statistical differences of shoot Cd and LMWOAs concentration between chicory and plantain were analysed with an unpaired t-test for each Cd treatment in the hydroponic experiment. A set of Pearson simple linear correlation analyses relating the xylem sap Cd concentration and shoot Cd concentration to different xylem sap LMWOAs concentration was performed for the hydroponic experiment. Stepwise regression was

used to determine overall relationships between xylem sap LMWOAs, hydroponic solution Cd and shoot Cd concentration in the hydroponic experiment.

5.5 Results and Discussion

5.5.1 Experiment one: Hydroponic experiment

5.5.1.1 Biomass dry matter

The average root dry weight of chicory for the 0.01, 0.1 and 0.5 mg Cd/L treatments was nominally lower than the control but significantly decreased ($P<0.05$) by 78% and 86% for the 2.5 and 5 mg Cd/L treatments, respectively, relative to the control (Table 5.2). The shoot dry weight of chicory did not show any significant ($P>0.05$) difference for the 0.01 mg Cd/L treatment, but significantly ($P<0.05$) decreased by 48%, 42% 85% and 90% for the 0.1, 0.5, 2.5 and 5 mg Cd/L treatments, respectively, compared to control (Table 5.2). In contrast, there was no significant difference in plantain root and shoot dry weight with increasing Cd concentration in the hydroponic media. This may be due to the lower Cd accumulation in plantain shoots than chicory shoots (discussed in the next section). Many studies have reported that high Cd accumulation in plant roots and shoots can cause plant growth reduction due to Cd toxicity (Ouzounidou et al., 1997; Xin et al., 2014; Huang et al., 2019). For example, Dias et al. (2013) reported that lettuce plants exposed to 0.1 and 1 mg Cd/L concentrations showed a significant decrease of 16 and 46% dry weight, respectively, compared to plants growing in control treatments. These authors suggested that plants growing in Cd-enriched solutions can uptake the metal through their roots, which accumulates in different tissues, eventually reducing plant growth and productivity via interfering with a number of normal metabolic processes. These may include (1) synthesis of proteins (2) the activities of some important enzymes by binding to free

amino, carboxylate or side groups and/or replace some important metal ions associated with such groups and (3) various photosynthetic processes such as chlorophyll biosynthesis (Stobart et al., 1985; Van Assche et al., 1988).

Table 5.2. Effect of Cd concentration in hydroponic media on chicory and plantain growth.

Added Cd concentration in the hydroponic solution (mg Cd/L)	Plant dry weight (g/plant DW)			
	Chicory		Plantain	
	Root	Shoot	Root	Shoot
0 (control)	5.90±1.50a	28.09±0.58a	3.54±0.04a	23.72±1.55a
0.01	4.98±0.78ab	21.88±1.82ab	2.59±0.05a	30.76±1.93a
0.1	2.89±0.36abc	14.40±1.83c	2.30±0.20a	27.88±2.22a
0.5	4.07±1.04abc	16.20±2.24bc	2.76±0.47a	23.96±0.79a
2.5	1.29±0.37bc	4.14±0.39d	2.08±0.37a	26.41±2.37a
5.0	0.80±0.09c	2.65±0.38d	2.04±0.19a	22.60±1.87a

Data are means±standard errors of three replicates. Values in each line, followed by different letters within a column for each plant, are significantly different at $P<0.05$ (n=3).

5.5.1.2 Plant tissue Cd concentration and translocation

The concentration of Cd in roots and shoots of both plant species was affected by the Cd concentration in hydroponic solution (Figure 5.2). There was a trend of increasing Cd concentration in roots and shoots of both chicory and plantain as the Cd concentration in solution increased from the control to 5 mg Cd/L (Figure 5.2). The shoot and root Cd concentration in chicory increased from 3.2 to 168.6 mg Cd/kg DW and 2.1 to 786.6 mg Cd/kg DW, respectively when the hydroponic solution Cd concentration increased from control to 5 mg Cd/L. The shoot and root Cd concentration in plantain increased from 2.5 to 54.6 mg Cd/kg DW and 8.0 to 1397.5 mg Cd/kg DW, respectively, when the hydroponic solution Cd concentration increased from control to 5 mg Cd/L level. In a similar study by Simon et al. (1996) the chicory shoot Cd concentration increased from 1.3 to 307.0 mg Cd/kg and the root Cd concentration from 1.0 to 891.1 mg Cd/kg when the hydroponic Cd concentration increased from the control treatment to 6 mg Cd/L in

the current work. The shoot Cd concentration of chicory was between 28 to 208% higher than for plantain for all Cd treatments (Appendix 4). This is in agreement with Abe et al. (2008) who investigated Cd uptake of 93 plants, including chicory and plantain, grown in sandy loam soil (3 mg Cd/kg soil) and who recorded a higher shoot Cd concentration in chicory (77 mg Cd/kg DW) than plantain (5.5 mg Cd/kg DW). A glasshouse study described by Stafford et al. (2016) found the mean tissue Cd concentration in chicory to be 231% higher than plantain.

In the current experiment the ratio of the Cd concentration in shoots to roots, defined as the Cd translocation factor, was calculated to describe the relative ability of these plants to translocate Cd from roots to shoots (Jeyakumar et al., 2014; Ubeynarayana et al., 2021). The TF of chicory significantly ($P<0.05$) increased from 1.5 to 3.5 when the Cd concentration increased from the control treatment to 0.01 mg Cd/L. The TF decreased from 3.5 to 0.2 with increasing Cd concentration from 0.01 to 5 mg Cd/L, however, this decrease was significant ($P<0.05$) only at 2.5 and 5 mg Cd/L hydroponic concentration compared to control (Figure 5.2a). Simon et al. (1996) observed that the TF factor of chicory significantly decreased from 1.1 to 0.3 when the hydroponic solution Cd concentration increased from the control treatment to 6 mg Cd/L. In the current work, the TF for plantain was always <1, with a decrease from 0.39 to 0.03 as the hydroponic Cd concentration increased from the control level to 5 mg Cd/L. However, this decrease was only significant ($P<0.05$) for a hydroponic Cd concentration of 0.01 mg Cd/L and greater (Figure 5.2b). These results showed that chicory had a higher TF range (12-4%) in all Cd treatment levels than plantain (Figure 5.2).

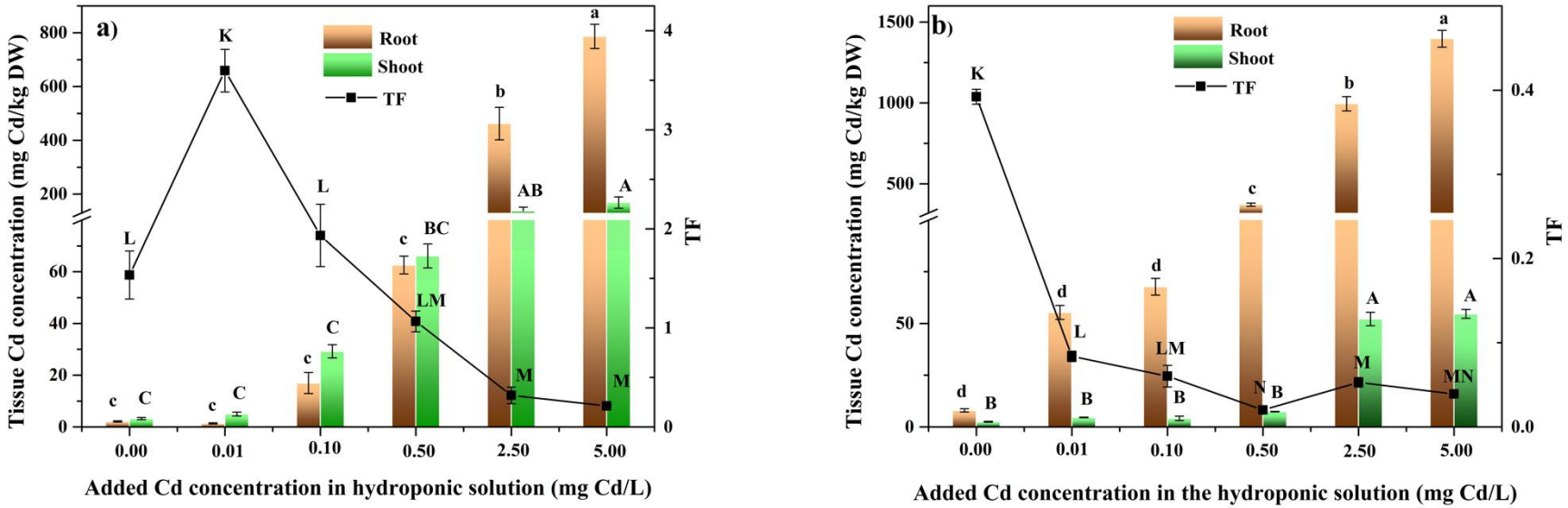


Figure 5.2. Cadmium concentration and TF of (a) chicory (b) plantain grown in different Cd concentrations in the hydroponic medium. Significant differences of root and shoot Cd concentrations between Cd treatments are represented by lower- (a-d) and upper- (A-D) case letters, respectively. Values in each bar followed by different letters are significantly different at $P<0.05$. Values in TF line followed by different letters (K-N) are significantly different at $P<0.05$. Vertical error bars represent ±SE (n=3).

5.5.1.3 Total and free xylem sap Cd concentration

The xylem sap Cd concentration in chicory and plantain showed a significant positive correlation (R=0.977, P<0.001; R=0.738, P<0.001, respectively) with hydroponic Cd concentration and increased from 0.1 to 6.4 mg Cd/L and 0.3 to 4.0 mg Cd/L, respectively when the hydroponic Cd concentration increased from control to 5 mg Cd/L (Figure 5.3). This increase was significant (P<0.05) for chicory for all hydroponic Cd concentrations of 0.1 mg Cd/L. However, for plantain, the xylem sap Cd concentration was not different for the 0.01 and 0.1 mg Cd/L treatments relative to the control, and higher Cd treatments.

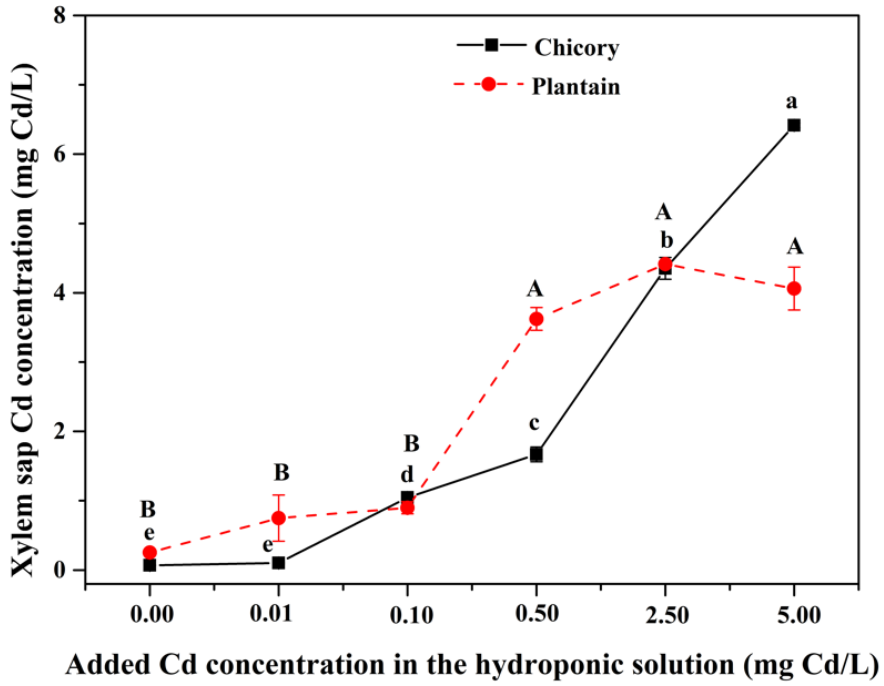


Figure 5.3. Xylem sap Cd concentration of chicory and plantain grown with increasing hydroponic Cd concentration. Significant differences of xylem sap Cd concentrations among Cd treatments of chicory and plaintain are represented by lower- and upper-case letters, respectively. Values in line followed by different letters are significantly different at P<0.05 (n=3). Vertical error bars represent ±SE (n=3).

Analysis of the free Cd^{2+} ion concentration in the xylem sap of chicory and plantain for the 5 mg Cd/L hydroponic Cd concentration treatment showed that 8% and 7% of Cd was present as the free Cd^{2+} ion, respectively (Table 5.3). Similarly, the free Cd^{2+} ion concentration of the 2.5 mg Cd/L hydroponic Cd concentration treatment showed that 9% and 10% of Cd in chicory and plantain xylem sap was present as the free Cd^{2+} ion, respectively. These results suggest that Cd^{2+} ions in the plant xylem sap are mainly in the complexed form.

Table 5.3. Free Cd^{2+} ion concentration of chicory and plantain xylem saps.

Sample name	**Free [Cd^{2+}] measured from electrode (mg Cd/L)**	**Total [Cd] measured from GFAAS (mg Cd/L)**
Plants grown in 5 mg Cd/L		
Chicory xylem sap	0.51±0.27	6.40±0.03
Plantain xylem sap	0.28±0.30	4.00±0.30
Plants grown in 2.5 mg Cd/L		
Chicory xylem sap	0.39±0.18	4.35±0.10
Plantain xylem sap	0.41±0.11	4.41±0.03

Data are means±standard errors of three replicates (n=3).

5.5.1.4 Composition and concentration of LMWOAs in xylem sap

The composition and quantities of LMWOA in the xylem sap of both plant species varied as the Cd concentration of the hydroponic solution increased from 0 to 5 mg Cd/L. Oxalic, fumaric, acetic, citric and malic acids were quantified as the major LMWOAs in xylem sap for all Cd treatments of both plants (Figure 5.4), and with the exception of fumaric acid, chicory produced significantly ($P<0.05$) higher concentrations of all LMWOAs than plantain under all Cd treatments (Figure 5.4).

The concentration of oxalic, acetic and citric acids in chicory xylem sap did not significantly vary for treatments up to 2.5 mg Cd/L, but significantly increased from 257.6 to 537.8 mg/L, 1237.7 to 2619.8 mg/L, and 266.5 to 716.5 mg/L, respectively, at the 5

mg Cd/L treatment compared to control (Figure 5.4). The fumaric acid concentration in chicory showed nominal variation between the control, 0.01 and 0.1 mg Cd/L treatments, but significantly ($P<0.05$) increased from 31.9 to 77.5, 31.9 to 55.9 and 31.9 to 68.1 mg/L at the 0.5, 2.5 and 5 mg Cd/L treatments, respectively, compared to the control. The malic acid concentration in chicory xylem sap did not show any significant ($P>0.05$) difference between the control, 0.01 and 0.1 mg Cd/L treatments but significantly ($P<0.05$) increased from 513.8 to 771.2 mg/L at the 0.5 mg Cd/L treatment and showed a nominal variation between the 0.5, 2.5 and 5 mg Cd/L treatments compared to control treatment. For plantain, the oxalic acid concentration in xylem sap did not significantly ($P>0.05$) change with increasing Cd levels in hydroponic solution and ranged from 18.8 to 37.1 mg/L. The acetic acid concentration in plantain xylem sap showed a nominal variation up to 2.5 mg Cd/L treatment and significantly increased by 88% (329.1-621.1 mg/L) at the 5 mg Cd/L treatment compared to the control. The concentration of citric acid (30.7-71.9 mg/L), fumaric acid (109.3-15.1 mg/L) and malic acid (62.7-158.8 mg/L) in plantain xylem sap did not show any trend with the increasing concentration of Cd in the hydroponic solution.

Studies have suggested that the complexation of Cd with LMWOAs will create an important pathway to avoid the toxicity of free reactive Cd^{2+} ions inside the plant, and reduce the impact of free Cd on plant metabolic processes including growth (Pence et al., 2000; Wei et al., 2007). In the current study, although the xylem sap LMWOA concentration increased with increasing hydroponic Cd concentration, there was a significant ($P<0.05$) reduction in chicory shoot biomass at the 0.1, 0.5, 2.5 and 5 mg Cd/L treatments compared to the control. Although there was a continuous biomass reduction with increasing Cd treatments, the reduction of biomass was only 42-48% up to 0.5 mg Cd/L treatment compared to the control and was associated with a nominal increase in the shoot Cd concentration. However, at 2.5 and 5 mg Cd/L treatments, there was a more

severe shoot biomass reduction (>80%) compared to the control with a significant increase in shoot Cd concentration. This observation implies that although xylem sap LMWOA production increased with increasing Cd concentration in hydroponic solution, these xylem sap LMWOAs may be limited in their Cd detoxification potential to only low concentrations of Cd in the growth media (i.e. the 0.01 mg Cd/L treatment) and only partially effective for Cd detoxification at medium-level Cd concentrations in the growth media (i.e. 0.1 and 0.5 mg Cd/L treatments). These results indicate that there is no effect of LMWOA in Cd detoxification at the solution concentration treatments of 2.5 and 5 mg Cd/L.

Some studies have shown that once absorbed, toxic metals are not completely inert and can interfere with the activities of specific enzymes of the plant respiration system and disrupt the TCA cycle leading to higher production of LMWOAs (Bansal et al., 2002; Mnasri et al., 2015). Greater shoot Cd accumulation in chicory reported in this work for the high Cd treatments may specifically impact the activity of the malate dehydrogenase enzyme in the TCA cycle inhibiting the conversion of malic acid to oxaloacetate. By this mechanism, the inhibition of oxaloacetate production in the TCA cycle will increase the production of other LMWOAs. Bansal et al. (2002) reported that Cd toxicity decreased the activity of malate dehydrogenase in pea seeds by 40% compared to control for a hydroponic media Cd concentration of 28 µg Cd/L. López-Millán et al. (2009) found that the activity of fumarase in extracts from tomato leaves decreased by 50% at a treatment level of 11.2 mg Cd/L compared to the control, and this reduction was attributed to Cd toxicity. However, further investigations should be carried out to confirm these explanations.

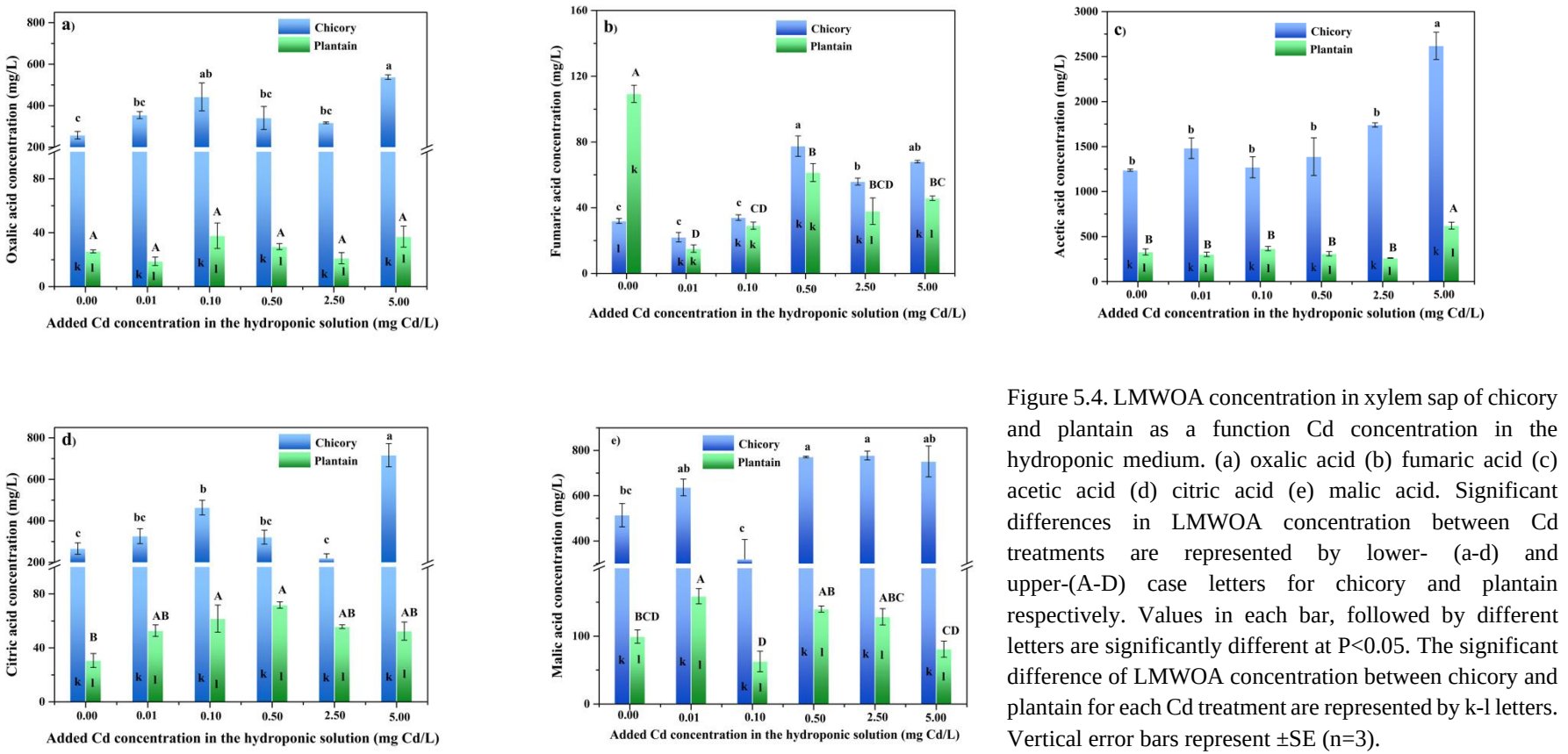


Figure 5.4. LMWOA concentration in xylem sap of chicory and plantain as a function Cd concentration in the hydroponic medium. (a) oxalic acid (b) fumaric acid (c) acetic acid (d) citric acid (e) malic acid. Significant differences in LMWOA concentration between Cd treatments are represented by lower- (a-d) and upper-(A-D) case letters for chicory and plantain respectively. Values in each bar, followed by different letters are significantly different at P<0.05. The significant difference of LMWOA concentration between chicory and plantain for each Cd treatment are represented by k-l letters. Vertical error bars represent ±SE (n=3).

5.5.1.5 Relationship of xylem sap and plant shoot Cd concentrations with xylem sap LMWOAs

The results presented in this chapter suggest that the increase of shoot Cd concentration with increasing Cd treatment levels in both plants may be due to the increase of bioavailable Cd concentration in the hydroponic solution. The shoot Cd concentration of chicory ($R=0.916$; $P<0.001$) and plantain ($R=0.927$; $P<0.001$) was significantly and positively correlated with the hydroponic Cd concentration and the shoot Cd concentration of both chicory ($R=0.942$, $P<0.001$) and plantain ($R=0.795$, $P<0.001$) showed a strong significant positive correlation with xylem sap Cd concentration. This implies that long-distance translocation of Cd via xylem is a key factor determining Cd accumulation in the above-ground part in chicory and plantain.

Many studies have identified LMWOAs as potential chelators to facilitate trace metal transport in plant species via xylem sap (Fu et al., 2019; Tao et al., 2020). To further investigate the factors that control the xylem sap and shoot tissue Cd concentration in chicory and plantain, Pearson's correlation coefficients were calculated for each factor (hydroponic solution Cd concentration and xylem sap LMWOA concentrations) (Table 5.4). Xylem sap and shoot tissue Cd concentrations in chicory significantly and positively correlated with all LMWOA concentrations, in xylem sap, and with the hydroponic solution Cd concentration ($R=0.606$-0.977 for xylem sap Cd; $R=0.606$-0.916 for shoot Cd). However, for plantain, the xylem sap and shoot tissue Cd concentration was only significantly correlated with the hydroponic solution Cd concentration ($R=0.738$ for xylem sap Cd; $R=0.927$ for shoot Cd). These data suggest xylem sap LMWOA does not influence the Cd uptake for plantain.

Table 5.4. Correlations coefficients (R) between xylem sap LMWOA concentrations with xylem sap Cd and shoot Cd concentration in chicory and plantain.

	Oxalic	Fumaric	Citric	Acetic	Malic	Solution [Cd]
Chicory						
Xylem sap [Cd]	0.764**	0.648*	0.721**	0.853**	0.606*	0.977**
Shoot tissue [Cd]	0.606*	0.700*	0.607*	0.753**	0.650*	0.916**
Plantain						
Xylem sap [Cd]	0.043	-0.157	0.360	0.232	0.130	0.738**
Shoot tissue [Cd]	0.084	-0.021	0.043	0.450	-0.121	0.927**

*P<0.05 **P<0.001

In order to determine which of these properties is the main factor controlling xylem sap or shoot tissue Cd concentration in chicory, a stepwise regression analysis was conducted using these factors. The regression analysis showed that the Cd concentration in the hydroponic solution and fumaric acid concentration in xylem sap were the controlling factors, explaining 96% and 88% of the variability of xylem sap and shoot tissue Cd concentrations, respectively. The stepwise regression equations are presented as Equations 1 and 2.

$$Xylem\ sap\ Cd\ concentration = -0.022 + 1.1411\ [Hydroponic\ Cd] + 0.01523\ [Fumaric] \quad \textbf{Equation 1}$$

$$Shoot\ Cd\ concentration = -8.9 + 27.19\ [Hydroponic\ Cd] + 0.838\ [Fumaric] \quad \textbf{Equation 2}$$

where, [Hydroponic Cd] = Hydroponic solution Cd concentration and [Fumaric] = Fumaric acid concentration in xylem sap.

Fumaric acid is a dicarboxylic acid and has a greater affinity towards Cd^{2+} ions (fumaric acid-pKa1=3.02, pKa2=4.44) (Adeniji et al., 2010). Cornu et al. (2020) found that fumaric acid production in xylem sap influenced Cd translocation via xylem sap in Sunflowers (*Helianthus*-ES RICA variety). Similarly, Tatár et al. (1998) reported that xylem sap fumaric acid production significantly increased the shoot Pb concentration of cucumber (*Cucumis sativus*).

5.5.2 Experiment two: Pot experiment

Results from the pot experiment showed there was no influence of LWMOA concentration or composition on the xylem sap or shoot Cd concentration in the plantain. Therefore, only the results for the chicory are further analysed and presented in this section. The results for plantain are presented in Appendix 5.

5.5.2.1 Soil and plant Cd concentration

The average total and bioavailable Cd concentration in the rhizosphere soil varied between the three soil types (Table 5.5). The Allophanic soil showed significantly higher total Cd concentration (0.71 mg Cd/kg) than the total Cd concentration in Gley (0.48 mg Cd/kg) and Recent soil (0.22 mg Cd/kg), respectively. The bioavailable Cd concentration followed the same trend where the Allophanic soil showed the highest concentration (0.20 mg Cd/kg) followed by the Gley (0.14 mg Cd/kg) and Recent soils (0.02 mg Cd/kg). These results agree with the earlier work of Stafford et al. (2018) who reported the mean soil Cd concentration of Allophanic soil (1.27 mg Cd/kg) to be higher than the mean Cd concentration in Gley soil (0.36 mg Cd/kg) (Table 5.5).

The Cd concentration in chicory shoots as a function of soil Cd concentration decreased following the order Allophanic> Gley> Recent, with on average Cd concentrations of 3.20, 1.91 and 0.94 mg Cd/kg DW, respectively (Table 5.4). The root Cd concentration followed the same trend with the greatest root Cd concentration recorded for the Allophanic soil (2.1 mg Cd/kg), followed by the Gley (1.2 mg Cd/kg) and Recent (1.1 mg Cd/kg) soils (Table 5.4). The xylem sap Cd concentration in chicory was highest for plants grown on the Allophanic soil (0.42 mg Cd/L) and considerably higher than for plants growing on the Gley (0.03 mg Cd/L) and Recent soils (0.04 mg Cd/L). The results

suggest that the significantly higher total and bioavailable Cd concentration in the Allophanic soil relative to the Gley and Recent soils had a significant influence on both the tissue and xylem sap Cd concentration in chicory plants.

Table 5.5. Summary of soil and plant Cd concentrations for chicory grown on three soil types.

Soil and Plant Cd concentrations (mg Cd/kg)	**Soil types**		
	Allophanic	**Gley**	**Recent**
Soil Total Cd	0.71±0.04a	0.48±0.05b	0.22±0.01c
Soil bioavailable Cd	0.20±0.04a	0.14±0.03b	0.02±0.00c
Plant root Cd	2.11±0.04a	1.25±0.07ab	1.11±0.04b
Plant shoot Cd	3.20±0.61a	1.91±0.06b	0.94±0.06c
Xylem sap Cd (mg/L)[1]	0.42±0.02a	0.03±0.00b	0.04±0.01c

Data are means±standard errors of three replicates. Values in each line, followed by different letters within a row for each parameter, are significantly different at $P<0.05$ (n=3). [1] Measured by GFAAS.

5.5.2.2 Composition and concentration of LMWOAs in xylem sap

The concentration of LMWOAs in the xylem sap of chicory varied as a function of soil type (Figure 5.5). LMWOAs measured in the xylem sap of chicory in the pot experiment were similar to the LMWOAs (acetic, citric, fumaric, malic and oxalic acids) measured in chicory plants from the hydroponic experiment, however, the concentrations were different. Only plants grown in the Allophanic soil showed a similar concentration of LMWOAs (with the exception of fumaric acid) to the hydroponic experiment. Chicory plants grown in the Gley and Recent soils produced lower LMWOA concentrations relative to all treatments of the hydroponic experiment (Figure 5.5).

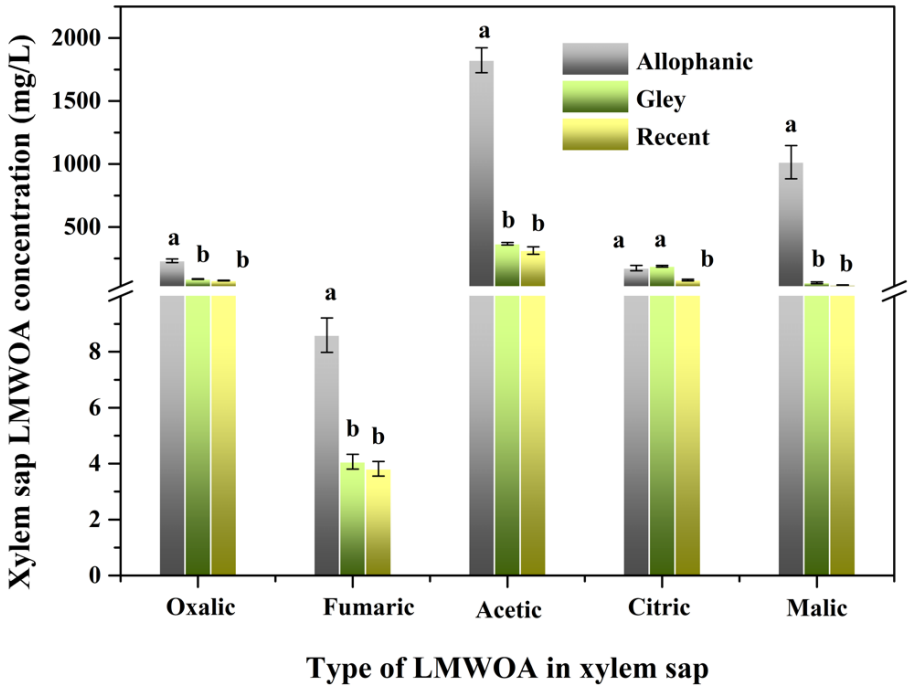


Figure 5.5. Concentrations of LMWOAs in xylem sap of chicory grown in three soil types. The significant difference of each LMWOA concentration between soil types is represented by a-d letters. Values in each bar, followed by different letters are significantly different at $P<0.05$. Vertical error bars represent ±SE (n=3).

5.5.2.3 Application of the regression model to predict shoot Cd concentration of chicory grown in different soil types

The shoot Cd concentration of chicory grown in the Allophanic, Gley and Recent were calculated using the equation developed in section 5.5.1.5. When the bioavailable soil Cd concentration in the Gley and Recent soils was used to predict the shoot Cd concentration using Equation 2, the calculated shoot Cd concentration values were negative due to low soil bioavailable Cd concentrations. To overcome this limitation the relationship between total soil Cd and bioavailable Cd was examined. The total soil Cd concentration showed a strong significant and positive correlation with bioavailable Cd concentration (R=0.944

P<0.001) (Figure 5.6). and therefore, the total soil Cd concentration was used to predict the shoot Cd concentration.

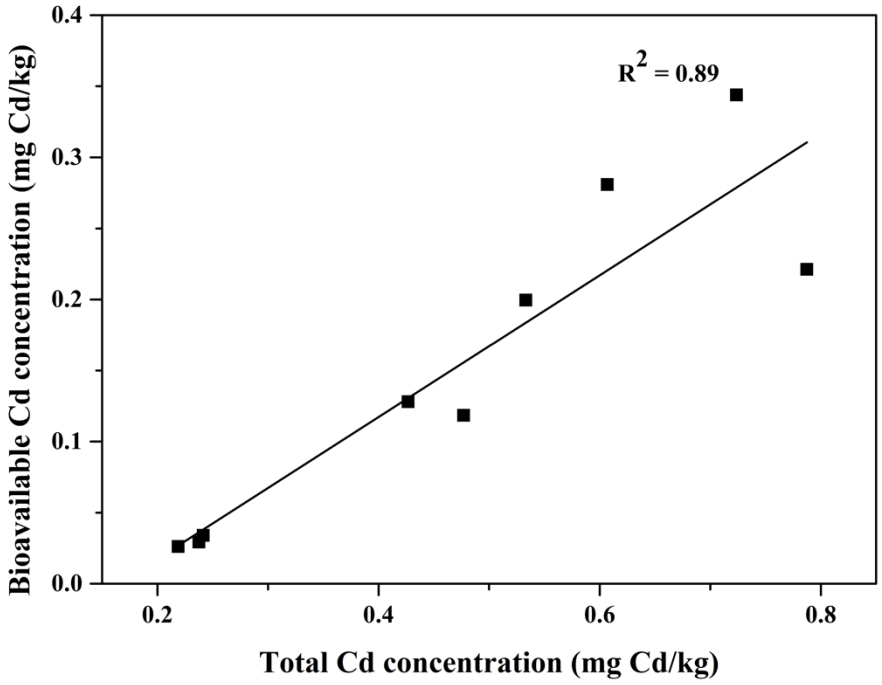


Figure 5.6. Relationship between total soil Cd concentration and bioavailable Cd concentration of three soil types.

The actual and predicted shoot Cd concentration of chicory grown in the three soil types are shown in Figure 5.7. The predicted shoot Cd concentration showed a significant and positive correlation (R=0.925, P<0.001) with the actual shoot Cd concentration for each soil type. However, the results showed that the model overpredicted shoot Cd concentration by factors of 5, 4 and 3 for the Allophanic, Gley and Recent soils, respectively. The reason for this higher prediction may be the use of 'total Cd concentration of soil' instead of 'soil bioavailable Cd concentration' of each soil type to predict the shoot Cd concentration in the model.

The confirmation of the specific role and active range of LMWOA concentration on Cd translocation will be tested only for chicory and discussed in the next chapter as LMWOA in plantain xylem sap does not influence Cd translocation in plantain.

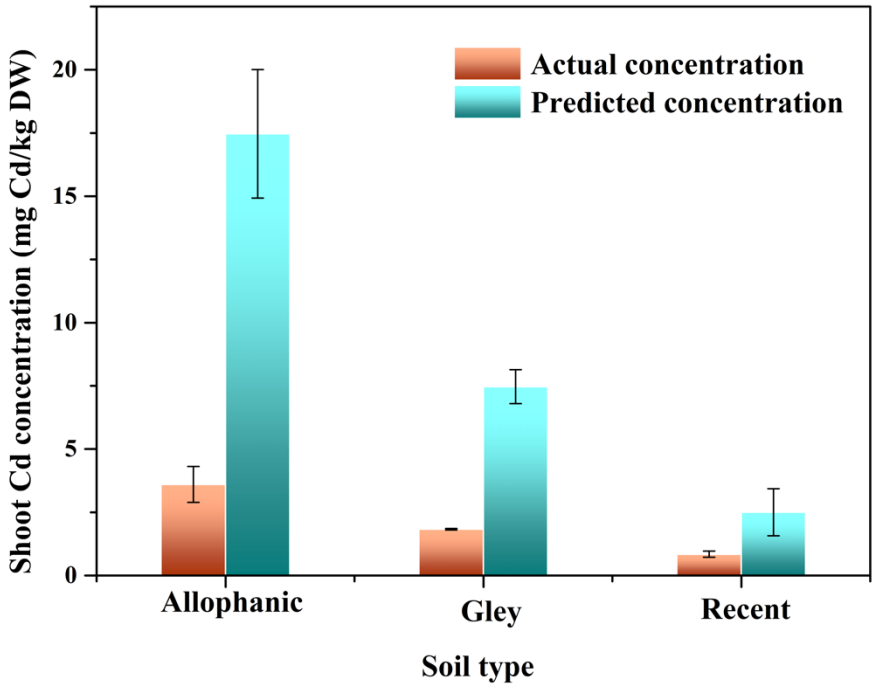


Figure 5.7. The predicted and actual shoot Cd concentration of chicory grown in different soil types. Vertical error bars represent ±SE (n=3).

5.6 Summary

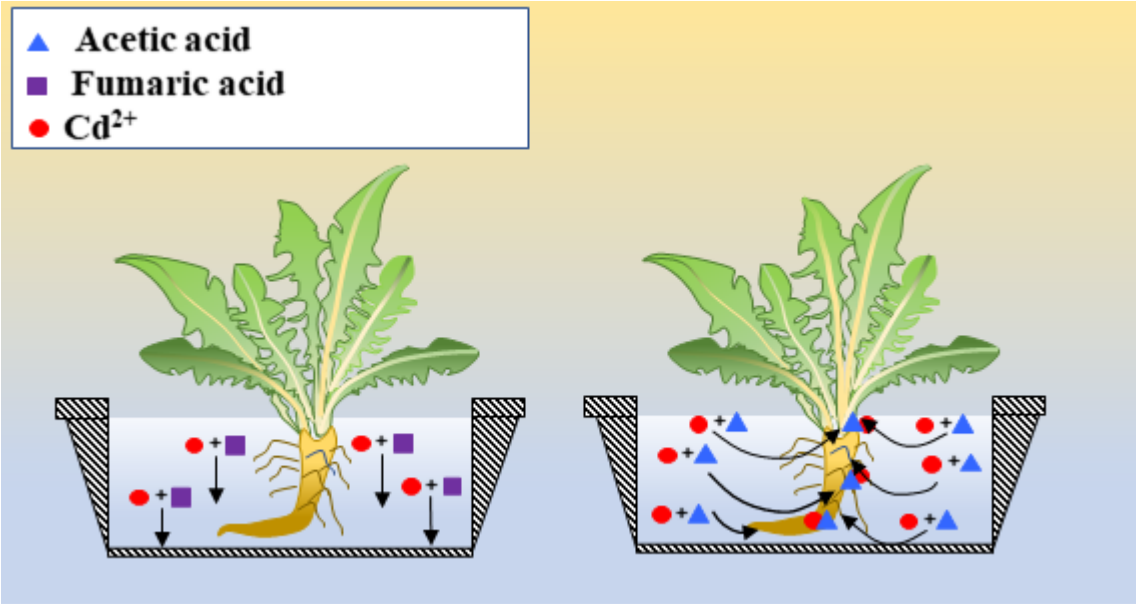
The results of this experiment showed that the quality and quantity of LMWOA in the xylem sap of chicory and plantain varies under different Cd treatments. Chicory produced a higher concentration of LMWOAs (oxalic, acetic, citric and malic) in the xylem sap except for fumaric acid compared to plantain. The free Cd^{2+} analysis of the xylem sap using the thiosalicylic acid-modified electrode showed Cd in the xylem sap of chicory and plantain existed dominantly in a complexed form than free Cd^{2+} ion form. There was

a significantly higher shoot Cd concentration in chicory than plantain for all Cd treatments and this difference can be explained in terms of variations of xylem sap LMWOA production in both plants. The regression analysis between shoot Cd concentration and xylem sap LMWOA concentration showed that Cd mainly binds with fumaric acid in chicory and translocate towards above-ground parts, while there was no significant association of LMWOAs on shoot Cd accumulation in plantain. However, reduction of chicory shoot biomass of chicory with increasing hydroponic Cd concentration suggests that although chicory produced a higher concentration of LMWOA with increasing Cd concentrations, these xylem sap LMWOAs may be limited in their Cd detoxification potential to only low concentrations of Cd in the growth media (i.e. the 0.01 mg Cd/L treatment).

Chapter 6

Effect of exogenous organic acid on Cd uptake and translocation in chicory

6.1 Graphical Abstract



6.2 Abstract

Low molecular weight organic acids play an important role in Cd uptake, translocation, and detoxification in forage plants such as chicory which are of economic importance to New Zealand agriculture. A hydroponic experiment was conducted to evaluate the effects of external application of fumaric, acetic and citric acids to a hydroponic solution on the uptake and translocation of Cd in chicory plants. Seedlings were grown for 21 days in a nutrient solution containing increasing concentration ratios of LMWOA to Cd: control, 1:0 (Cd-only), 1:1, 10:1, 50:1, 100:1 for each LMWOA. The entire experiment was replicated, with the nutrient solution in one replicate renewed every 7 days and the

nutrient solution in the other replicate maintained without renewal for the duration of the 21 days of the experiment. Analysis of the hydroponic solution showed that the efficacy and stability of LMWOA decreased as a function of time. An effect of LMWOA was only observed in treatments that were renewed every 7 days. Fumaric acid reduced Cd uptake and translocation in chicory with a maximum reduction achieved at a ratio of 10:1 and 50:1 fumaric acid to Cd for root and shoot Cd accumulation, respectively. Acetic acid significantly increased the shoot Cd concentration at lower acetic acid levels (1:1 treatment) and reduced the shoot Cd accumulation with increasing acetic acid concentrations from 10:1 to 100:1 treatment ratio. Root Cd accumulation increased for the 1:1 to 50:1 treatment. There was no effect of citric acid on Cd uptake and translocation at any treatment ratio. The current work found no strong ameliorative effect of LMWOA on Cd toxicity at any concentration for the LMWOA ratios and Cd concentration (1 mg Cd/L) used in this study.

6.3 Introduction

Among the various LMWOA produced by plants, studies have identified acetic acid as a potential ligand for Cd which can increase the Cd concentration in plants. For example, Hawrylak-Nowak et al. (2015) reported that the addition of acetic acid to growth media at an acetic acid to Cd concentration ratio of 100:1 increased the root Cd concentration in sunflower from 378.5-572.0 mg Cd/kg DW, compared to a Cd-only treatment (0.5 mg Cd/L). These authors also observed a significant increase in root FW by 42% relative to the Cd-only treatment suggesting that the complexation of Cd with acetic acid may alleviate Cd toxicity in roots. Similarly, Han et al. (2006) showed that maximum root Cd accumulation in maize plants was achieved through increasing the acetic acid: Cd concentration ratio in the hydroponic solution from 1:1 to 50:1. Root Cd accumulation

then decreased with a further increase in organic acid to above a Cd ratio of 100:1. Han et al. (2006) suggested that either acetic-Cd complexes in the soil/hydroponic solution can act as a carrier for Cd^{2+} ions towards the root surface and that these complexes can disassociate into free Cd^{2+} at the root surface which is absorbed by root membrane, or mobile organically bound Cd-complexes can penetrate via root membrane to increase Cd uptake. Cieśliński et al. (1998) suggested that higher root secretion of acetic acid from wheat cultivar-Kyle relative to wheat cultivar -Arcola, contributed to high Cd uptake and translocation in Kyle. These authors observed that higher secretion of acetic acid (by 163%) in Kyle significantly increased both shoot and total plant Cd content by 153% and 33%, respectively, compared to Arcola for plants growing in Sutherland sandy loam soil with a total Cd concentration of 0.41 mg/kg.

Plants also have mechanisms associated with fumaric acid to regulate root Cd uptake and translocation (Tatár et al., 1998; Kazemi Movahed, 2020). For example, Oloumi et al. (2011) reported that the addition of fumaric acid (5 mg/L) to the growing media significantly ($P<0.05$) reduced the total Cd concentration in canola seedlings in the presence of 1 mg Cd/L compared to the control treatment (reduction was by 98%). Fan et al. (2016) suggested that a significant ($P<0.05$) increase of fumaric acid secretion from 1.2 to 2.0 mg/L in rice (Oryza sativa cultivar Hua-Hang-Si-Miao) exposed to a Cd + Si (5 mg Cd/L + 42 mg Si/L) treatment influenced the chelation of Cd^{2+} ions and reduced plant Cd uptake relative to the control. Kazemi Movahed (2020) observed a higher secretion of fumaric acid (11-fold compared to control) by low Cd accumulating soya bean cultivar (AC Hime) roots and lower secretion of fumaric acid (3-fold compared to control) in high Cd accumulating soya bean cultivar (Westag 97) at a treatment concentration of 3.3 mg Cd/L. These authors suggested that fumaric acid reduced the solubility and bioavailability of Cd for uptake by the plant through 1) the formation of

Cd-LMWOA complexes in the growing media, or 2) steric factors associated with complexes that are too large to cross root membranes easily preventing Cd influx into the root cells. However, the effect of fumaric acid on increasing root Cd uptake and root sequestration has not been well documented in the literature.

In addition to root Cd uptake, studies have reported that increasing metal concentration in the growing media increases the production of fumaric acid in the plant xylem sap and can influence both the xylem plant Cd translocation and shoot Cd accumulation (Tatár et al., 1998; Cornu et al., 2020). For example, Cornu et al. (2020) found that fumaric acid production in xylem sap influenced Cd translocation via xylem sap in Sunflowers (ES RICA variety).

Literature has also identified citric acid as a ligand for Cd uptake and translocation in plants (Senden et al., 1995; Ehsan et al., 2014; Wang et al., 2017). For example, Senden and Wolterbeek (1990) reported that metal complexes, such as Cd-citrate, in tomatoes, could transport Cd efficiently from root to shoot via xylem sap. A study by Ehsan et al. (2014) reported that the addition of citric acid (480 mg/L) to Cd (5.6 mg Cd/L) significantly increased the shoot Cd concentration by 31% compared to a non-citric acid control. However, the dose of citric acid is important. Li et al. (2014) reported that a low dose (288 mg/L) of citric acid induced a 26.7% higher concentration of Cd in ramie root compared to a higher dose (786 mg/L) in the presence of 10 mg /L Cd. In contrast to these reported increases of root Cd uptake associated with citric acid, Pinto et al. (2008) reported that under Cd stress, citrate exuded from the roots of maize and sorghum effectively decreased the free Cd ion concentration in solution, and this, in turn, reduced Cd uptake by maize and sorghum plants. Literature evidence suggests that different plants

have specific mechanisms to regulate Cd uptake and translocation, and these mechanisms may be associated with different concentrations of different types of LMWOAs.

Previous experiments in this thesis suggested the lower secretion of fumaric acid and higher secretion of acetic acid was associated with an increase in root Cd uptake by chicory, while fumaric acid production in xylem sap facilitated translocation of this metal within chicory plants. However, the literature reported in this introduction has also identified citric acid as a common carrier for Cd uptake and translocation in many plants. Therefore, the relative impact of fumaric, acetic and citric acids on Cd uptake and translocation to aerial tissues in chicory deserves more research. This chapter reports the findings of an experiment which investigated the effect of external application of fumaric, acetic and citric acid on plant Cd uptake and translocation in chicory. The stability of LMWOAs in the environment (Hoagland solution) with time was also examined as this parameter will assist the assessment of using LMWOAs in pastoral agriculture to regulate Cd uptake. The specific objectives of this study were (a) to explore the specific role of fumaric, acetic and citric acid on Cd uptake and translocation in chicory and their effect on the alleviation of Cd toxicity in chicory; and (b) to understand the stability of fumaric, acetic and citric acid in the environmental media with time.

6.4 Materials and Methods

6.4.1 Hydroponic experiment

A hydroponic experiment was set up in a greenhouse at the Massey University Plant Growth Unit with average day/night temperatures of 17/20 ℃. Growth media was a modified Hoagland solution adjusted to six increasing concentration ratios (mg/L) of LMWOA to Cd: 0:0 (control), 0:1(Cd-only), 1:1, 10:1, 50:1, and 100:1 with fumaric,

acetic, and citric acids, independently. The concentrations (mg/L) of Cd and each LMWOA in these treatments are shown in Table 6.1. Plants grown in Hoagland solutions without organic acid and Cd (i.e. treatment 0:0) were used as controls.

Table 6.1. The concentration of Cd and LMWOA in each LMWOA treatment.

LMWOA: Cd concentration ratio in the treatments	**Cd concentration in the treatment (mg/L)**	**LMWOA concentration in the treatment (mg/L)**
Control (0:0)	0.0	0.0
0:1	1.0	0.0
1:1	1.0	1.0
10:1	1.0	10.0
50:1	1.0	50.0
100:1	1.0	100.0

The composition of the Hoagland solution was: 602.2 mg/L (3 mM) $Ca(NO_3)_2 \cdot 2H_2O$, 303.3 mg/L (3 mM) KNO_3, 123.3 mg/L (0.5 mM) $MgSO_4 \cdot 7H_2O$, 68.1 mg/L (0.5 mM) KH_2PO_4, 16.2 mg/L (0.1 mM) $FeCl_3$, 0.6 mg/L (10 μM) H_3BO_3, 0.2 mg/L (1 μM) $MnCl_2 \cdot 4H_2O$, 0.1 mg/L (0.5 μM) $ZnSO_4 \cdot 7H_2O$, (1 μg/L) 0.01 μM H_2MoO_4 and 0.02 mg/L (0.1 μM) $CuSO_4 \cdot 5H_2O$. The $FeCl_3$ was substituted for EDTA-Fe in the Hoagland solution to prevent possible chelating reactions with Cd. The pH of the solution was adjusted to 5.5-6.0 every day using 0.1M HNO_3 acid to prevent significant depletion of nutrients and changes in pH. Chicory was used in this experiment, and germination and the experimental set up were similar to the experimental procedures explained in Chapter 5 (Figure 6.1). Hydroponic containers were arranged in a completely randomized design and growth was continued for 21 days. The entire experiment was replicated, with the Hoagland solution in one replicate renewed every 7 days (Hoagland solution replacement on Day 7 and Day 14 hereafter described as the 'renewed' replicate treatment). The Hoagland solution in the other replicate was maintained without renewal for the 21 days of the experiment ('non-renewed' replicate treatment). In a parallel experiment, change of the hydroponic solution pH and LMWOA concentration of the 1:1, 10:1, 50:1 and

100:1 LMWOA: Cd treatments (all fumaric, acetic and citric treatments) were measured as a function of the experiment days. More details of this experiment are presented in Appendix 6.

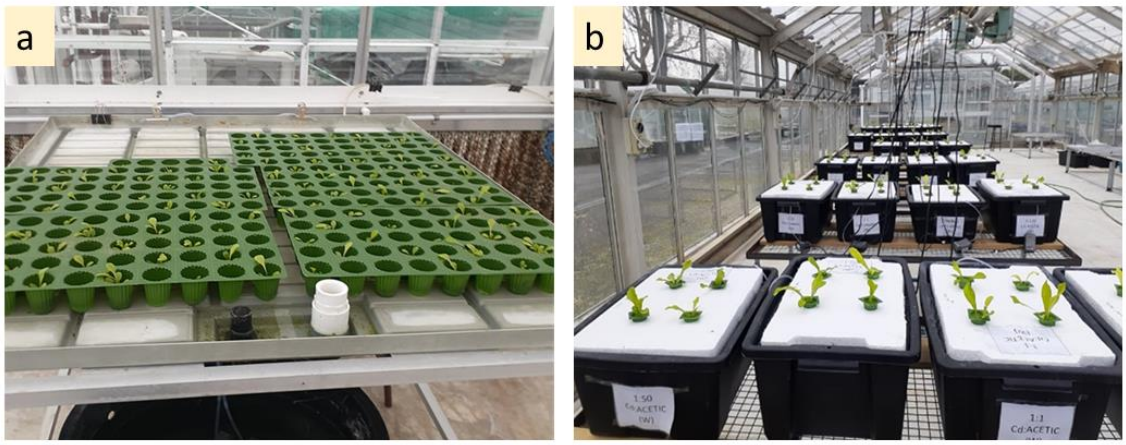


Figure 6.1. Experimental setup in the greenhouse. (a) Germination of the chicory seeds in green plastic cups on germination bench (b) arrangement of the hydroponic containers in the greenhouse.

6.4.2 Plant harvest

Twenty-one days after transplanting, plant shoots and roots were removed separately from nutrient media and roots were immediately dipped in a cold 0.36×10^{-3} mg/L HCl solution to remove any Cd adsorbed to the root surface (Ghnaya et al., 2013).

6.4.3 Chemical analysis

Quantification of plant shoot and root dry weight, tissue Cd concentration, and LMOWA concentrations in the hydroponic solution were performed as explained in Chapter 5. The pH of the hydroponic solution was measured using a Eutech Instruments Cyber Scan pH 310. The shoot to root translocation factor (TF) was calculated as the ratio of shoot Cd

concentration to root Cd concentration (Jeyakumar et al., 2010). The growth tolerance index (TI) of the shoot and root of plants grown in different LMWOA treatments was calculated as the ratio between the shoot or root dry weight at treatment and the shoot/root dry weight at control (Huang et al., 2019).

6.4.4 Transmission electron microscope observation of shoot cells

Shoot samples (approximately 5 mm in length) from the Cd-only, 100:1 fumaric, 100:1 acetic and 100:1 citric acid treatments collected (at the end of the experiment) from the renewed (weekly) hydroponic replicate were fixed with 3 % (v/v) glutaraldehyde and 2% (v/v) formaldehyde in 0.1 M phosphate buffer (pH 7.2) for at least 2 hr. Fixed samples were buffer washed 3 times in 0.1 mol/L phosphate buffer (pH 7.2) for 10 min each before being post-fixed in 1% Osmium Tetroxide in 0.1 mol/L phosphate buffer for no more than 1 hr. Samples were then again buffer washed 3 times (as above) for 10 min each and dehydrated using a graded acetone series (25%, 50%, 75%, 95%, and 100%) for 10-15 min each followed by 2x changes of 100% for 1 hr each. The dehydrated samples were placed into 50:50 resin: acetone and stirred overnight. The leaf samples were slowly infiltrated with resin over 3 days before being placed into silicon moulds with fresh resin and cured for 48 hr to give rectangular blocks that could be cut with a microtome. The block of resin containing the leaf sample was then trimmed down to the selected area using a Diamond Knife (Diatome, Austria) set at 100 nm. These were stretched with chloroform to prevent wrinkle and mounted on a grid using a Quick Coat G glue pen (Saiko, Japan). The 100 nm sections mounted on grids were stained in Saturated Uranyl Acetate in 50% ethanol for 4 min, washed with 50% ethanol and MilliQ water and then stained in lead citrate for a further 4 min. This was followed by a wash in MilliQ water.

Samples were viewed using an FEI Tecnai G2 Spirit BioTWIN Transmission Electron Microscope (Czech Republic).

6.4.5 Quality control measures

All chemicals used were of analytical grade. The limit of detection for Cd in this work was 0.002 mg Cd/L. The accuracy of the measurements was assessed by analysing certified reference materials in parallel with unknown samples. For plant total tissue Cd analysis, NIST 1573a (tomato leaves; National Institute of Standards and Technology) was used as certified reference material. The analysed Cd concentration was within 94-108% of the expected mean value (1.52 mg Cd/kg).

6.4.6 Statistical analysis

Statistical analysis was conducted with Minitab 18 and OriginPro 9 (Origin Lab, USA) statistical software. The effect of Cd treatments on different plant and soil variables was statistically analysed using a one-way ANOVA test; if a significant ($P<0.05$) main effect was detected, the difference between treatment means was tested using a Tukey HSD posthoc test. The significant differences of the shoot and root Cd concentrations between renewed and non-renewed treatments were analysed with an unpaired t-test for each type of Cd: LMWOA treatment.

6.5 Results and Discussion

6.5.1 Composition of the hydroponic solution

6.5.1.1 Variation of LMWOA with time

The concentration of LMWOAs in the hydroponic solution significantly reduced ($P<0.05$) as a function of increasing days of the experiment for all treatments (Figure 6.2). The fumaric acid concentration in solutions decreased by 100%, 100%, 87% and 81% for the 1:1, 10:1, 50:1 and 100:1 treatment, respectively with time from Day 1 to 7. The corresponding decrease for the acetic acid and citric acid treatments was 100% for all treatment combinations. The reduction of LMWOA concentration with time may be a result of the microbial degradation of the LMWOAs or LMWOA-Cd complexes with time: organic acids represent one of the most labile sources of carbon in the hydroponic media and therefore it is possible that microorganisms will metabolise this form of exogenous carbon (Jones et al., 2003).

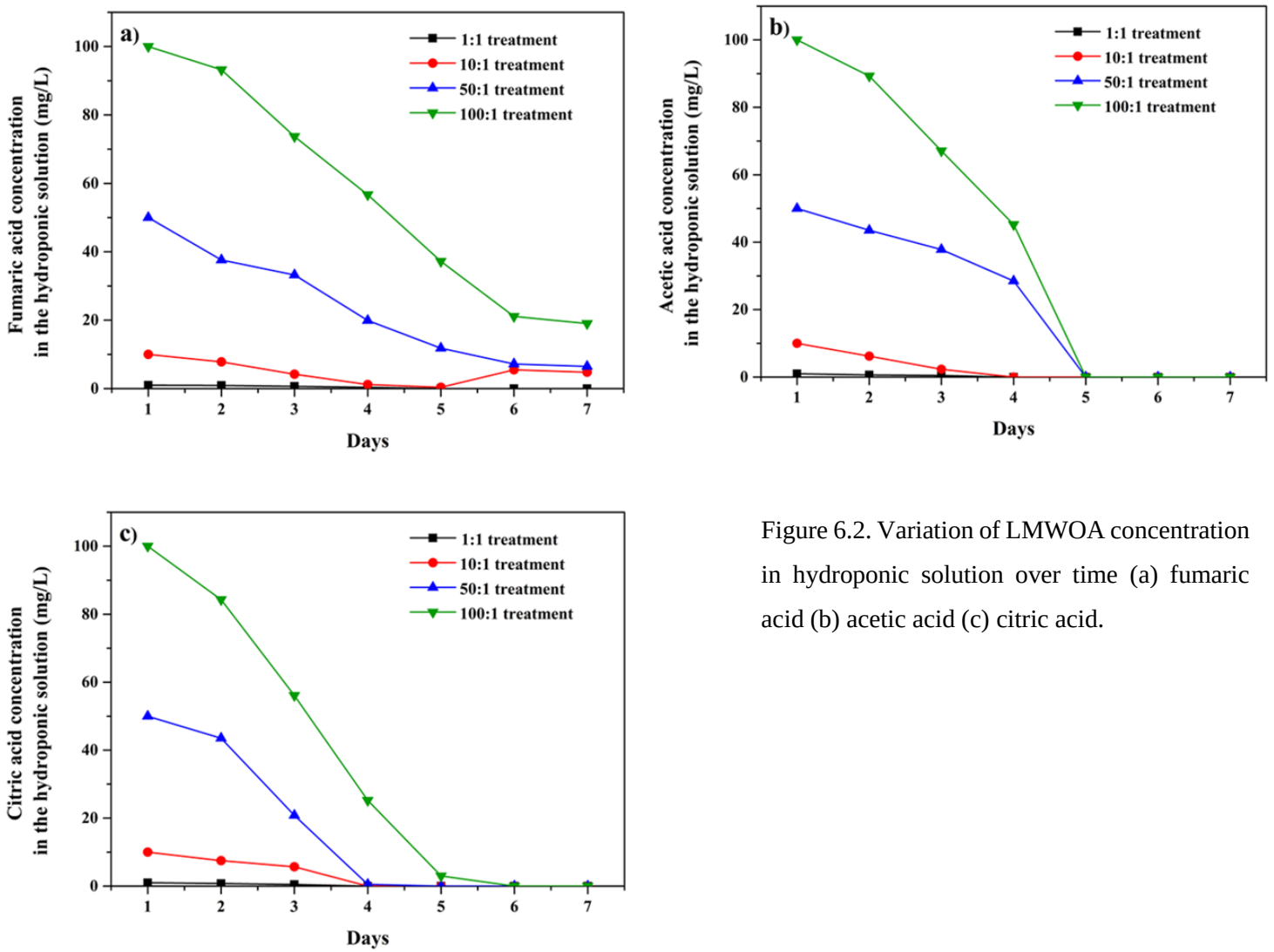


Figure 6.2. Variation of LMWOA concentration in hydroponic solution over time (a) fumaric acid (b) acetic acid (c) citric acid.

6.5.1.2 Variation of pH with time

The pH of the hydroponic solution was initially significantly reduced for all LMWOA treatments compared to the control treatment (Figure 6.3). However, after Day 1, the pH of the hydroponic solution increased each day. The initial pH drop may be due to the immediate dissociation of H^+ ions from the acid form of LMWOA added to the growth media (Osmolovskaya et al., 2018). Evangelou et al. (2008) reported that soil pH increased from 5.5 to 7.7 over 96 hrs for a Cu (450 mg Cu/kg) + citric acid (62.5 mmol/kg) soil treatment. They suggested that this pH increase resulted from the microbial degradation of carboxylic acids (LMWOA) which consumed H^+ and liberated OH^- and CO_2.

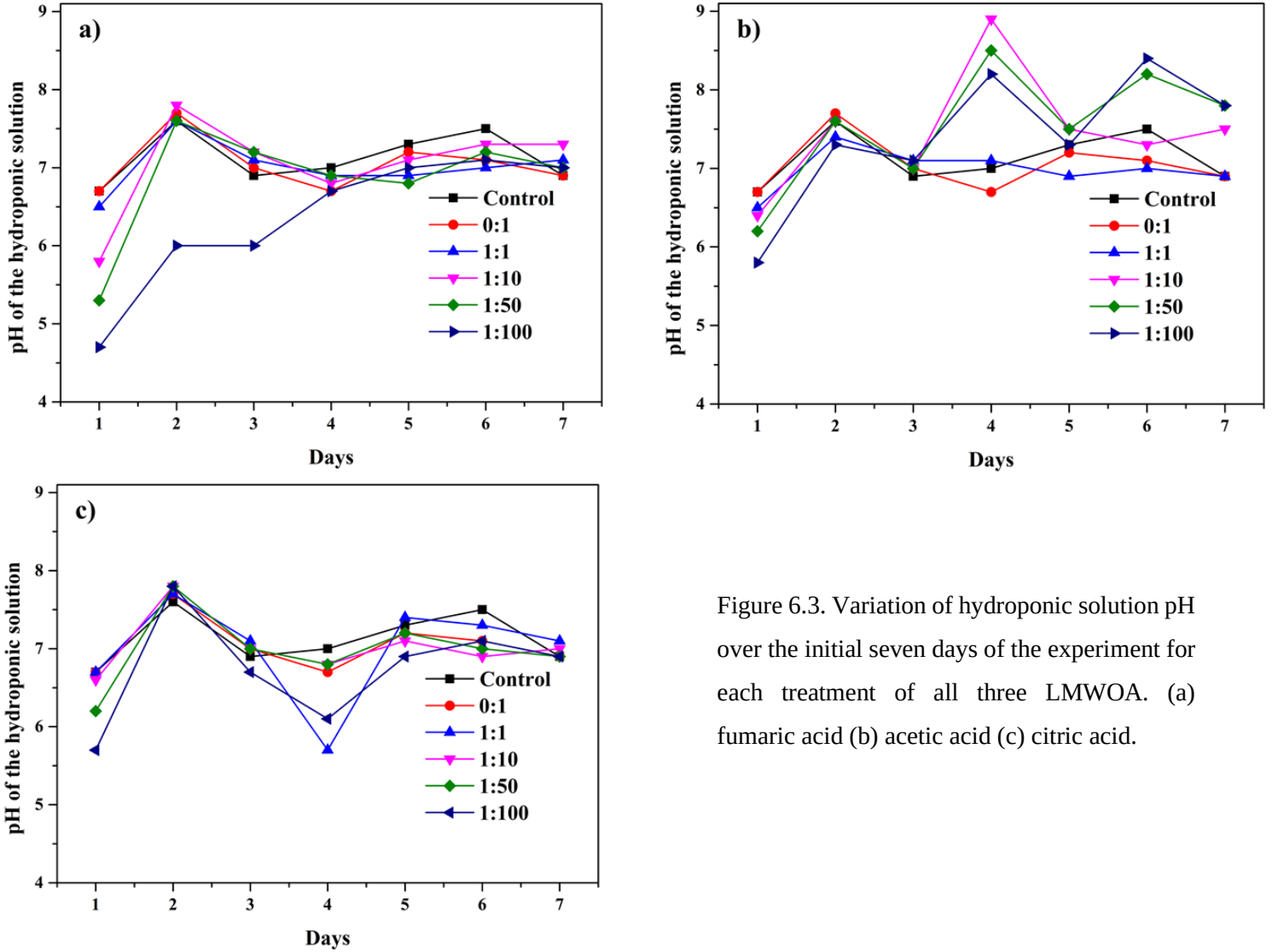


Figure 6.3. Variation of hydroponic solution pH over the initial seven days of the experiment for each treatment of all three LMWOA. (a) fumaric acid (b) acetic acid (c) citric acid.

6.5.2 Tissue Cd concentration

The Cd accumulation in the shoots and roots of chicory was affected by the external application of LMWOAs into the hydroponic solution and the frequency of solution (treatment) renewal (Figure 6.4).

The shoot Cd concentration decreased as a function of increasing fumaric acid concentration in the hydroponic solution from the 1:1 to 50:1 fumaric acid treatment ratio with the weekly renewal of fumaric acid treatments (Figure 6.4a). However, this decrease was significant ($P<0.05$) only at fumaric acid to Cd ratios of 10:1 and 50:1 where the reduction in tissue Cd concentration was by 37% and 36%, respectively, relative to the Cd-only treatment (weekly renewed). However, the shoot Cd concentration significantly ($P<0.05$) increased by 34% for the 100:1 fumaric acid treatment compared to the Cd-only treatment. The root Cd concentration also decreased by 22% and 32% for the 1:1 and 10:1 fumaric acid treatments, respectively, compared to the Cd-only treatment, but significantly ($P<0.05$) increased by 43% and 75% at 50:1 and 100:1 fumaric acid treatments, respectively, compared to Cd-only treatment (Figure 6.4a).

The root and shoot Cd concentration of plants grown in the non-renewed fumaric acid treatments showed no significant difference ($P<0.05$) among treatments. However, there was a nominal decrease in root Cd concentration by 10%, 17% and 30% at the 10:1, 50:1 and 100:1 treatment ratio, respectively, relative to the Cd-only control, and a nominal decrease by 21%, 39% and 18% in the shoot Cd concentration for the 10:1, 50:1 and 100:1 treatments respectively, compared to Cd-only treatment (Figure 6.4a).

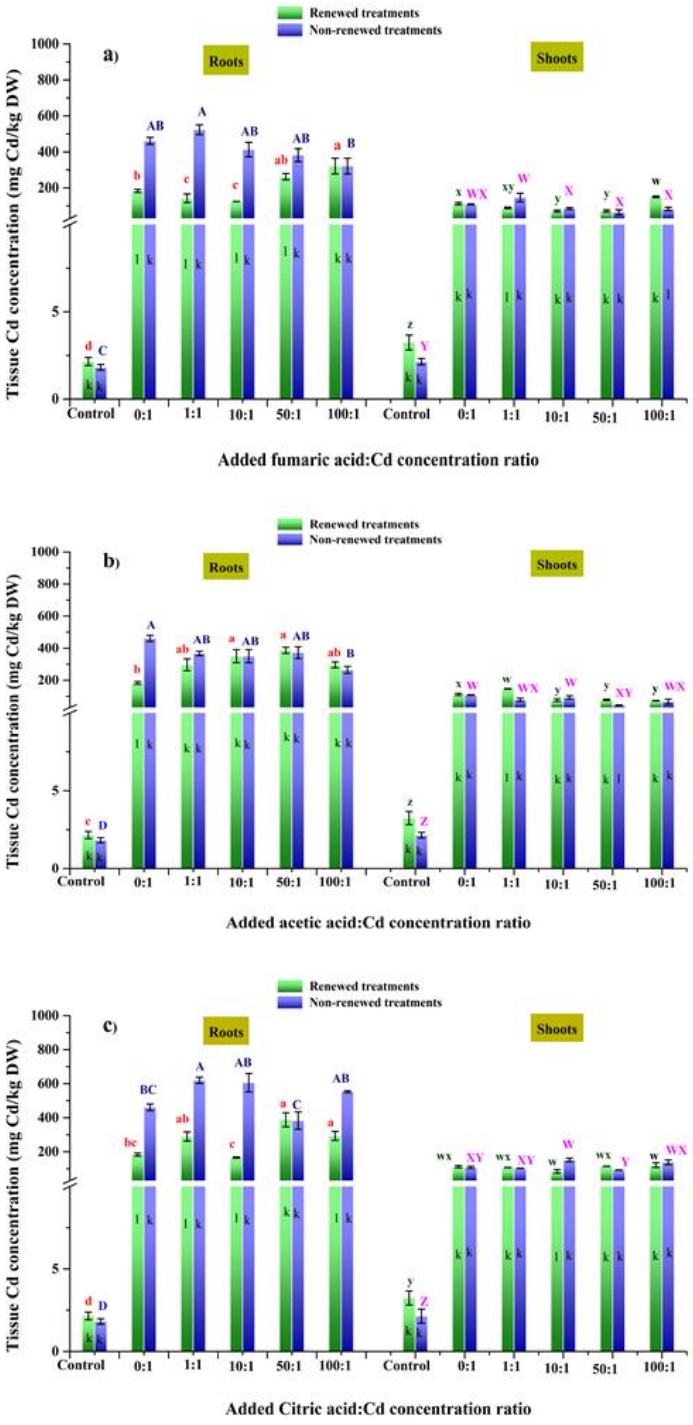


Figure 6.4. Shoot and root Cd concentration of chicory grown in different LMWOA concentrations in renewed and non-renewed hydroponic solution; (a) fumaric acid (b) acetic acid (c) citric acid. Significant differences (at P<0.05) of root Cd concentration between Cd treatments of plants grown in renewed and non-renewed hydroponic solutions are represented by lower- (a-d) and upper-case (A-D) letters, respectively.Significant differences (at P<0.05) of shoot Cd concentration between Cd treatments in renewed and non-renewed hydroponic solutions are represented by lower- (w-z) and upper-case (W-Z) letters, respectively. Significant differences (at P<0.05) of both root and shoot Cd concentration between the plants grown in renewed and non-renewed hydroponic solutions for each Cd treatment are represented by k-n letters, respectively Vertical error bars represent ±SE (n=3).

The shoot Cd concentration significantly increased ($P<0.05$) as a function of acetic acid treatment (by 31%) for the 1:1 treatment, and then significant ($P<0.05$) decreased by 32%, 30%, and 35% at 10:1, 50:1 and 100:1 treatments, respectively, relative to the Cd-only treatment where acetic acid was renewed weekly. In contrast to shoots, the root Cd concentration showed a trend of increasing Cd concentration with an increasing ratio of acetic acid treatment compared to the Cd-only treatment where acetic acid was renewed weekly. However, this increase was significant ($P<0.05$) only for the 10:1 and 50:1 acetic acid treatment, by 90% and 110%, respectively, compared to the Cd-only treatment (weekly renewed) (Figure 6.4b).

For the replicate design with no renewal of acetic acid treatment, there was no significant difference observed between root Cd concentration of chicory plants at acetic acid concentration ratios less than 100:1. The root Cd concentration significantly ($P<0.05$) reduced by 42%, at 100:1 acetic acid treatment, compared to the Cd-only treatment. However, for this replicate, where LMWOAs were not renewed weekly, the shoot Cd concentration showed a significant ($P<0.05$) decrease only at the 50:1 treatment by 59% compared to Cd-only treatment. The shoot Cd concentration for the 1:1, 10:1 and 100:1 acetic acid treatment did not show any significant ($P>0.05$) difference with the shoot Cd concentration of the Cd-only treatment (Figure 6.4b).

There was no significant difference ($P<0.05$) in shoot Cd concentration as a function of increasing hydroponic solution citric acid concentrations compared to the Cd-only treatment where citric acid was renewed weekly (Figure 6.4c). However, root Cd concentration significantly increased by 111% and 60% at 50:1 and 100:1 citric acid treatment, respectively, compared to the Cd-only treatment (weekly renewed). The shoot and root Cd concentration of chicory grown in non-renewed citric acid treatments did not

show any defined trend with increasing hydroponic citric acid concentration compared to the Cd-only treatment. However, the shoot Cd concentration showed a significant increase ($P<0.05$) as a function of citric acid treatment (by 40%) at the 10:1 treatment, although there was no significant difference observed between the shoot Cd concentration of 1:1, 50:1 and 100:1 treatments and Cd-only treatment (weekly non-renewed) where citric acid was not renewed weekly. The root Cd concentrations where citric acid was not renewed weekly, showed a significant increase (by 34%) at the 1:1 treatment compared to the Cd-only treatment. The root Cd concentration for the other treatments (1:1, 50:1 and 100:1) showed nominal variation with the root Cd concentration of the Cd-only treatment (Figure 6.4c).

6.5.2.1 Translocation of Cd from root to shoot

The TF of plants grown in fumaric acid treatments decreased as a function of increasing fumaric acid concentration in the hydroponic solution from the 1:1 to 50:1 fumaric acid treatment ratio where fumaric acid treatments were renewed weekly (Figure 6.5a). However, this decrease was significant ($P<0.05$) only at the 50:1 fumaric acid to Cd ratio where the reduction was by 55% relative to the Cd-only treatment (weekly renewed). The TF of plants grown in the non-renewed fumaric acid treatments did not show any significant ($P>0.05$) difference compared to the Cd-only treatment (Figure 6.5a). The TF of plants grown in acetic acid treatments decreased from 0.6 to 0.2 with the increase of treatment from the Cd-only treatment to the 100:1 treatment when the acetic acid was renewed weekly. This decrease was significant ($P<0.05$) for an acetic acid treatment ratio of 10:1 and greater (Figure 6.5b). Plants grown in the replicate design with no renewal of acetic acid treatment did not show any significant difference of TF among treatments (Figure 6.5b).

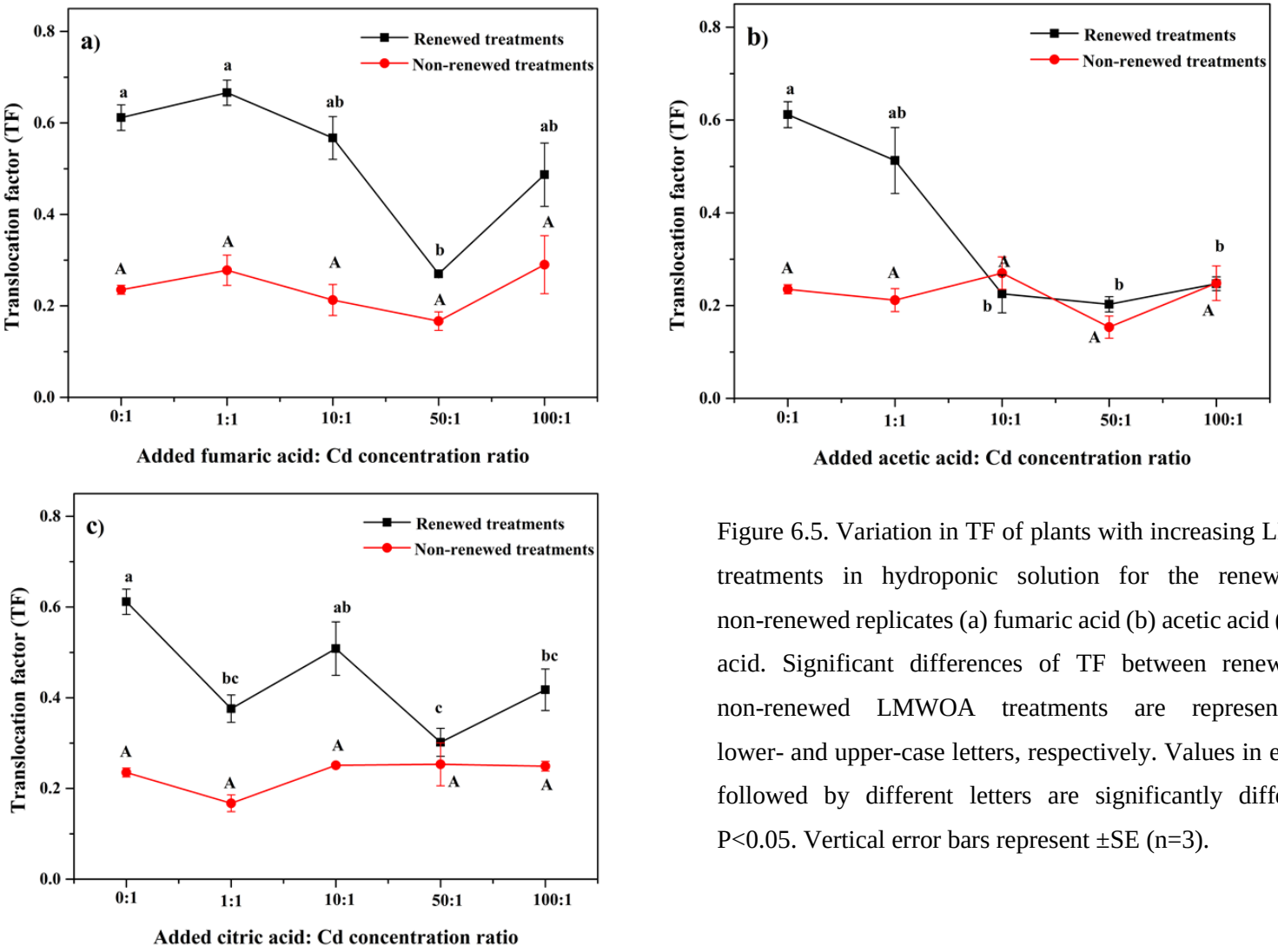


Figure 6.5. Variation in TF of plants with increasing LMWOA treatments in hydroponic solution for the renewed and non-renewed replicates (a) fumaric acid (b) acetic acid (c) citric acid. Significant differences of TF between renewed and non-renewed LMWOA treatments are represented by lower- and upper-case letters, respectively. Values in each line followed by different letters are significantly different at $P<0.05$. Vertical error bars represent ±SE (n=3).

There was no trend observed in plant TF (0.6-0.4) as a function of increasing hydroponic solution citric acid concentration compared to the Cd-only treatment where citric acid was renewed weekly (Figure 6.5c). The TF of plants grown in the non-renewed citric acid treatments did not show any significant difference ($P<0.05$) with increasing hydroponic citric acid concentration compared to the Cd-only treatment. These results show that the TF of plants significantly ($P<0.05$) varies with increasing LMWOA concentrations (especially acetic acid) when the LMWOA is weekly renewed. However, there was no significant ($P>0.05$) difference observed in TF of plants as a function of LMWOA where the treatment was not renewed weekly. This discrepancy may be due to the reduction of efficacy of LMWOA with increasing days of the experiment.

6.5.3 Biomass dry matter content

A significant increase in the shoot and root chicory Cd concentration was correlated with a significant reduction in the shoot and root dry weight of the plants (Table 6.2). Where the hydroponic solution was renewed weekly, the shoot dry weight significantly ($P<0.05$) decreased by 79% for the Cd-only treatment compared to the control (0 Cd treatment) while the root dry weight nominally decreased by 22% for the same treatment (Table 6.2). Where the hydroponic solution was not renewed, the reduction in shoot and root dry weight was by 66% and 41%, respectively, for the Cd-only treatment compared to the control (0 Cd treatment). With respect to Cd treatments with LMWOA, there was a difference in root and shoot biomass as a function of solution renewal. For treatments where hydroponic solution as replaced weekly, there was no effect of fumaric and citric acid treatments on the shoot and root dry weights relative to the Cd-only treatment. There was, however, an effect of weekly renewed acetic acid on shoot dry weight: the shoot dry weights of plants grown in 10:1, 50:1 and 100:1 weekly renewed acetic acid treatments

significantly ($P<0.05$) increased by 73%, 87% and 112%, respectively, compared to the Cd-only treatment. The significantly higher shoot dry weight production at high concentration (>10:1) of acetic acid treatments may be due to the significant reduction of shoot Cd concentration with increasing acetic acid concentration in the hydroponic solution. For the replicate design with no renewal of citric or acetic acid treatment, there was no significant difference observed between the root and shoot Cd concentration of chicory with increasing organic acid treatment levels compared to the Cd-only treatment. However, the shoot and root dry weight of plants grown in fumaric acid treatment, where LMWOAs were not renewed weekly, significantly ($P<0.05$) increased by 80% and 70% at 100:1 and 50:1 treatments, respectively, compared to Cd-only treatments.

Table 6.2.Effect of increasing LMWOA concentrations in hydroponic solution (1 mg Cd/L) on chicory growth for weekly renewed and non-renewed treatments.

[Organic acid]: [Cd] (mg acid/L: mg Cd/L)	Plant dry weight (g/plant)							
	Weekly renewed				Weekly non-renewed			
	Root	Root (TI)	Shoot	Shoot (TI)	Root	Root (TI)	Shoot	Shoot (TI)
Fumaric acid								
0:0 (control)	1.37±0.30a	1.00±0.00a	6.55±0.10a	1.00±0.00a	1.63±0.20a	1.00 ±0.02a	7.59±0.40a	1.00±0.01a
0:1 (Cd-only)	1.06±0.19a	0.77±0.04ab	1.34±0.30b	0.20±0.05b	0.95±0.02bc	0.58±0.09bc	2.53±0.06c	0.33±0.07c
1:1	1.12±0.05a	0.81±0.03ab	1.00±0.08b	0.15±0.01b	0.84±0.05bc	0.51±0.03c	1.58±0.14c	0.21±0.01c
10:1	0.65±0.18a	0.47±0.10b	1.40±0.05b	0.21±0.01b	0.65±0.11c	0.40±0.01c	1.55±0.21c	0.21±0.02c
50:1	1.23±0.18a	0.90±0.11ab	1.73±0.29b	0.26±0.04b	1.63±0.03a	1.00±0.01a	3.40±0.73bc	0.44±0.09bc
100:1	1.18±0.09a	0.86±0.06ab	1.39±0.04b	0.21±0.01b	1.33±0.15ab	0.82±0.09ab	4.58±0.10b	0.61±0.01b
Acetic acid								
0:0 (control)	1.37±0.3a	1.00±0.00a	6.55±0.10a	1.00±0.00a	1.63±0.2a	1.00±0.02a	7.59±0.40a	1.00±0.01a
0:1 (Cd-only)	1.06±0.01a	0.77±0.04b	1.34±0.30c	0.20±0.05d	0.95±0.02ab	0.58±0.09b	2.53±0.06b	0.33±0.07b
1:1	1.05±0.07a	0.76±0.04b	2.09±0.02bc	0.32±0.00c	0.50±0.06b	0.31±0.03b	1.71±0.21b	0.22±0.02b
10:1	1.08±0.11a	0.79±0.07ab	2.31±0.11b	0.35±0.01bc	0.80±0.06b	0.49±0.03b	2.47±0.06b	0.32±0.01b
50:1	1.09±0.03a	0.79±0.02ab	2.50±0.10b	0.38±0.01bc	0.67±0.03b	0.41±0.01b	1.11±0.20b	0.14±0.02b
100:1	0.79±0.07a	0.58±0.05b	2.84±0.02b	0.43±0.00b	0.62±0.28a	0.99±0.17a	1.62±0.60b	0.21±0.07b
Citric acid								
0:0(control)	1.37±0.3a	1.00±0.00a	6.55±0.10a	1.00±0.00a	1.63±0.20a	1.00±0.02a	7.59±0.40a	1.00±0.01a
0:1 (Cd-only)	1.06±0.01a	0.77±0.04ab	1.34±0.30b	0.20±0.02b	0.95±0.02b	0.58±0.09b	2.53±0.06b	0.33±0.07b
1:1	0.75±0.07a	0.54±0.04b	0.96±0.13b	0.14±0.02b	0.82±0.03b	0.50±0.02b	2.99±0.16b	0.39±0.02b
10:1	1.13±0.05a	0.82±0.03ab	1.28±0.08b	0.19±0.01b	0.83±0.05b	0.51±0.00b	2.47±0.73b	0.32±0.09b
50:1	1.11±0.21a	0.80±0.02ab	1.52±0.28b	0.39±0.10b	0.88±0.09b	0.54±0.02b	3.21±0.39b	0.42±0.05b
100:1	0.83±0.21a	0.60±0.01ab	1.34±0.27b	0.20±0.04b	0.81±0.09b	0.50±0.05b	2.34±0.27b	0.30±0.03b

Data are means±standard errors of three replicates. Values in each line, followed by different letters within a column for each LMWOA, are significantly different at $P<0.05$ (n=3).

6.5.3.1 Growth tolerance index

The shoot and root tolerance index is defined as the ratio of dry weight for plants growing in the Cd containing growing media and the dry weight for control plants (no Cd in the hydroponic solution) and was calculated to better explain the relative variations of plant dry weight due to Cd toxicity caused by Cd accumulation in plant tissues (Ali et al., 2002). For the plants grown in weekly renewed hydroponic solution, the shoot tolerance index significantly ($P<0.05$) reduced from 1.00 to 0.20 and the root tolerance index nominally decreased from 1.00 to 0.77 when the treatment varied from the control (0:0) to the Cd-only treatment (0:1). Similarly, for plants grown in the weekly non-renewed hydroponic solution, there was a significant ($P<0.05$) reduction of both shoot and root tolerance index from 1.00 to 0.33 and 1.00 to 0.58, respectively, when the treatment changed from control to the Cd-only treatment. The significant reduction ($P<0.05$) of root and shoot tolerance index in both weekly renewed and non-renewed Cd-only treatments may be due to Cd accumulation in plant tissues interfering with a number of normal plant metabolic processes and leading to a plant growth reduction (Xin et al., 2014; Huang et al., 2019).

To visual explore this finding, photomicrographs were obtained from TEM analysis of the shoot cells in the Cd-only treatment grown in weekly renewed hydroponic solution (Figure 6.6). The effect of Cd toxicity on the ultrastructure of shoot cells is visually confirmed by chloroplast swelling and cell wall damage in Figure 6.6a. There was an observable increase of plastoglobulus in the chloroplast stroma of the plant cells for the Cd-only treatment (Figure 6.6b). Studies have suggested that an increase of plastoglobulus is a symptom of Cd toxicity in plants since these globules are lipid droplets of degraded thylakoid membranes (de Araújo et al., 2017).

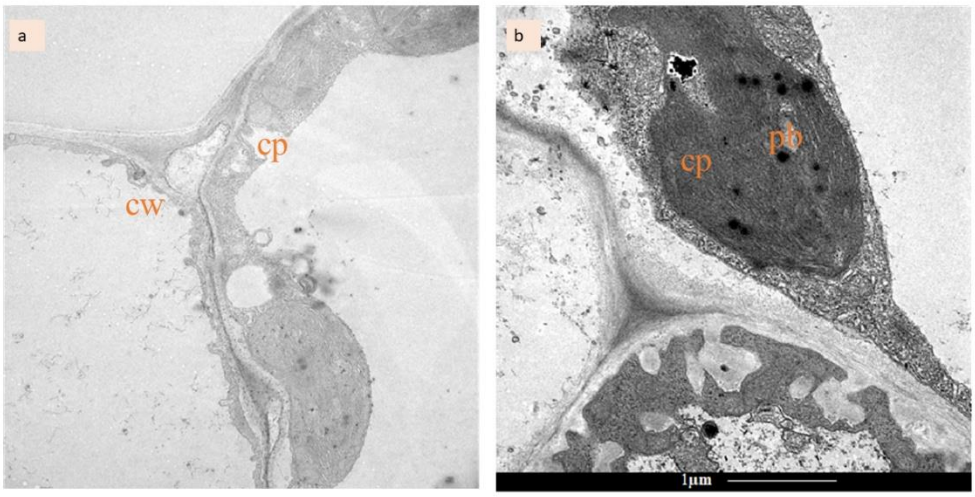


Figure 6.6. Transmission Electron Micrographs of shoot cells at Cd-only treatment. (a) chloroplast swelling and disruption at Cd-only treatment; (b) increase of plastoglobulus in the chloroplast stroma at Cd-only treatment. CW- cell wall, CP- Chloroplast, Pb- Plastoglobulus.

For treatments where hydroponic solution as replaced weekly, there was no effect of fumaric and citric acid treatments on the shoot and root tolerance index, or the root tolerance index of acetic acid treatments relative to the Cd-only treatment. The plants grown in weekly renewed acetic acid treatments showed a significant increase in the shoot tolerance index at 1:1, 10:1, 50:1 and 100:1 treatment by 60%, 72%, 86% and 111% compared to values for the Cd-only treatment. This may be due to the significant ($P<0.05$) reduction of shoot Cd concentration with increasing acetic acid treatments compared to the Cd-only treatment (see section 6.4.2). This is further confirmed by photomicrographs of TEM analysis which showed clear well- organized cell structures for 100:1 acetic acid weekly renewed treatment (Figure 6.7b). For, 100:1 weekly renewed fumaric treatment there was a chloroplast disruption was observed (Figure 6.7a). However, although there was no significant growth difference of shoot between Cd-only treatment and 100:1 citric

treatment, there was no significant effect of Cd toxicity observed in 100:1 citric acid shoot cell (Figure 6.7c).

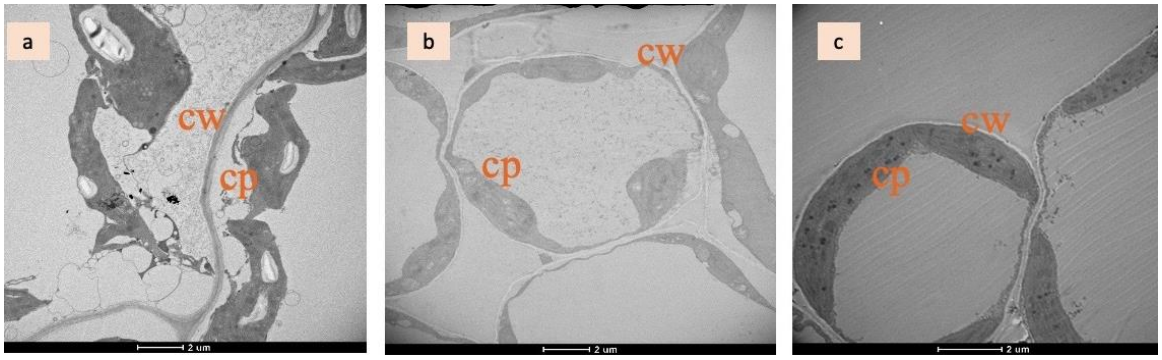


Figure 6.7. Transmission Electron Micrographs of shoot cells at different LMWOA treatments. (a) chloroplast disruption at 100:1 fumaric acid treatment; (b) well-organized cell structure at 100:1 acetic acid treatment; (c) well-organized cell structure at 100:1 citric acid treatment. CW- cell wall, CP- Chloroplast.

Plants grown in the replicate design with no renewal did not show any significant difference ($P>0.05$) in shoot and root tolerance index with increasing acetic and citric acid treatments compared to the Cd-only treatment. However, for weekly non renewed fumaric acid treatments, the shoot and root tolerance index significantly increased by 84% and 100% at the 100:1 and 50:1 treatment ratio, respectively, compared to the Cd-only treatment. The shoot and root Cd concentration results showed that there was no significant difference observed in the shoot (100:1 treatment) and root (50:1 treatment) Cd concentration with the Cd-only treatment shoot and root Cd concentration. These results are unlikely to be due to detoxification of Cd as a result of LMWOAs in solution due to the recorded high degradation rate of LMWOAs in solution with time as shown in Figure 6.2. Thus, this discrepancy may be due to an increase in nutrient (P) availability as a function of degradation of fumaric acid or the growth of microorganisms in the hydroponic solution which promote plant growth (Strobel, 2001; Jacoby et al., 2017).

Overall, these results show that there was very limited evidence for a strong ameliorative effect of LMWOA on Cd toxicity, even at very high concentrations of LMWOA.

6.5.4 Effect of LMWOAs on plant Cd uptake and translocation of chicory.

Plants which were grown in weekly renewed organic acid treatments had observable trends of the shoot and root Cd concentration variation and TF variation with increasing LMWOA treatments for all three organic acids (Figures 6.4 and 6.5). However, the plants grown in the weekly non-renewed LMWOA treatments did not show any clear trend in shoot and root Cd concentration variation and there were no significant differences observed in TF among treatments. This suggests that the effect of LMWOA was only apparent where organic acids were renewed weekly. This may be a consequence of microbial degradation of LMWOA throughout the experiment, reducing the efficacy of the treatment over time. The previous explanation of significantly higher microbial degradation of LMWOA with time in the hydroponic solution (see section 6.4.1.1) supports the minor effect of LMWOA on shoot and root Cd concentration of weekly non-renewed organic acid treatments. This suggests that the specific effect of fumaric, acetic and citric acid on the shoot and root Cd uptake and translocation may not be explained using the results obtained from weekly non-renewed LMWOA treatments. Thus, the results obtained from only the weekly renewed LMWOA treatments are used to evaluate the impact of LMWOAs on chicory root and shoot Cd accumulation here.

There was a significant reduction in shoot and root Cd concentration with increasing fumaric acid concentration in hydroponic solution up to the 50:1 treatment ratio. This observation implies that fumaric acid in hydroponic solution (up to 50:1 treatment) may complex with Cd ions in the hydroponic solution and prevent the penetration of Cd via

plant root membranes and reduce plant Cd uptake. Oloumi et al. (2011) reported that the addition of Cd: fumaric acid at a 1:5 concentration ratio to growth media significantly ($P<0.05$) reduced the total Cd concentration by 98% in canola seedlings compared to the control. Similarly, Kazemi Movahed (2020) found that an 11-fold increase in the secretion of fumaric acid by soya bean (cultivar AC Hime) at a treatment concentration of 3.3 mg Cd/L reduced Cd bioavailability and uptake by plants relative to the control. These authors suggested that LMWOA ligands in the growing media do not necessarily increase the solubility, transport and bioavailability of metals, but can reduce Cd transport and uptake either by the formation of complexes with Cd, or by decreasing of free Cd^{2+} ion concentration.

However, enhanced Cd accumulation in root and shoot tissues at high fumaric acid concentration levels in the current work (100:1 treatment) may be due to a decrease of hydroponic solution pH compared to other treatments in the initial days of the experiment (Figure 6.3a). High external LMWOA (including fumaric acid) levels in the hydroponic solution can cause changes in plant cell structures, leading to phytotoxicity in plants (Turgut et al., 2004). Najeeb et al. (2011) explained that the addition of higher concentrations of ligands (LMWOAs) into hydroponic solution can influence the shape and structure of plant cells and ATPases in the root plasma membrane which can change the mode of the transport of ions through the membrane and increase Cd uptake through symplasmic or apoplasmic pathways. In the context of the current study, this explanation was investigated by analysing TEM photomicrographs from the 100:1 fumaric acid treatment in shoot cells. The TEM images showed disrupted and swelled chloroplasts in the shoot cells grown at the 100:1 fumaric acid to Cd treatment ratio and this is a main symptom of phytotoxicity (Figure 6.7a).

The increase in root Cd accumulation from the 1:1 to 50:1 acetic acid treatments and decrease in shoot Cd accumulation from the 1:1 to 100:1 acetic acid treatments explains that Cd may concentrate in roots by binding as Cd-acetic complexes to cortex cell walls which limits the transfer of Cd from the root cortex into the stele. The root: shoot Cd concentration ratio showed a significant positive correlation ($R=0.729$ $P<0.05$) with acetic acid treatments (up to 50 mg/L treatment). Increased Cd accumulation in roots may serve to reduce Cd translocation from roots to shoots and reduce Cd toxicity in the shoot. The TEM images obtained for the 100:1 acetic acid treatment showed clear, well-organized shoot cell structures (Figure 6.7b). Han et al. (2006) reported that adding acetic acid (15 mg/L) to the hydroponic solution significantly increased the maize root Cd concentration by 125% in the presence of 0.56 mg Cd/L compared to a solution without acetic acid. They explained the high root Cd accumulation in maize via two mechanisms: (1) penetration of mobile and soluble organically-bound Cd complexes (acetic-Cd) via cell membranes to increase Cd uptake and accumulate in the root, (2) The ability of the acetic-Cd complex in the soil solution act as a carrier for Cd^{2+} ions towards the root surface and disassociation of these complexes into free Cd^{2+} at the root surface which can be absorbed by root membrane and accumulate in roots.

The increasing citric acid concentration in the hydroponic solution showed a limited effect on Cd accumulation in chicory roots and shoots except for significantly ($P<0.05$) higher Cd accumulation at high citric acid concentration treatments (50:1 and 100:1). Thus, suggests little evidence that citric acid influences root to shoot Cd translocation in chicory.

6.6 Summary

The results obtained from the present study demonstrated that external application of fumaric, acetic and citric acids, have limited practical impact on Cd uptake and translocation in chicory due to the decrease of efficacy and stability of LMWOA with increasing time. External application of fumaric acid showed the ability to reduce Cd uptake and translocation in chicory with a maximum reduction achieved at treatment ratios of 10:1 and 50:1 LMWOA to Cd for root and shoot Cd accumulation, respectively. Acetic acid treatments promoted Cd uptake and translocation in chicory. However, the maximum shoot Cd accumulation was observed at a ratio of 1:1 and shoot Cd accumulation significantly ($P<0.05$) reduced with increasing acetic acid treatments from 10:1 to 100:1. In contrast, root Cd accumulation increased with increasing acetic acid treatments from 1:1 and reached maximum concentration at the 50:1 treatment. There was a very limited effect of citric acid on Cd uptake and translocation in chicory. There was no strong evidence observed to explain the potential of LMWOAs to ameliorate Cd toxicity in chicory. This may be due to the low stability of LMWOA in hydroponic solution over time.

Chapter 7
Integrated discussion: Key findings, implications of the research and suggestions for future work

7.1 Background

Increased Cd accumulation in agricultural soils has been linked to risks for soil health and, as a result, to potential impacts on food production. In New Zealand, soil Cd concentrations have significantly increased in both pastoral and horticultural soil as the result of long-term phosphate fertiliser application (Loganathan et al., 2003; Salmanzadeh et al., 2017). For example, Waikato Regional Council has estimated that 11% of Waikato's pastoral soils and 17% of its horticultural soils have a total Cd concentration that exceeds the contamination threshold of 1 mg Cd/ kg soil (Stafford, 2017). Despite being a nonessential trace element, Cd can be absorbed by plant roots and transported to aerial parts (Senden and Wolterbeek, 1990). Hence Cd contamination of New Zealand's most versatile soils threatens to limit their use for high-value pasture, vegetable and tuber cropping due to the risk of Cd accumulation in the food chain (Reiser et al., 2014).

In 1990 new forage species were introduced to New Zealand's livestock grazing systems due to their high drought tolerance, nutrient content and environmental benefits. For example, chicory and plantain are deeper rooting plants than perennial ryegrass and clover and could be useful in reducing nitrate-N leaching losses from grazed pasture systems (Li and Kemp, 2005). However, research by Stafford et al. (2016) showed that forage species such as chicory and plantain can accumulate significantly higher Cd concentrations from even low Cd soils when compared to grasses and legumes, which have traditionally been used in New Zealand agriculture. Grazing Cd-rich forage has the modelled potential to

cause an exceedance of the maximum guideline level for Cd in the kidneys and livers of livestock (Lee et al., 1996), although the relative risks of such exceedance between forage crops are poorly understood.

To address this knowledge gap, the work for this doctoral thesis was designed and implemented to investigate the potential mechanism which may play a role in Cd uptake and translocation in chicory and plantain. The intended application of this work is to help develop strategies which could assist in avoiding high Cd accumulation in offal to maintain the standards of New Zealand's food production.

There have been no studies published on the Cd uptake mechanisms of forage species such as chicory and plantain used in New Zealand agricultural systems. A review of the literature at the outset of this doctoral study highlighted that different plant species have different mechanisms to absorb Cd from soils and that translocate of Cd from root to shoot is associated with LMWOAs (de la Luz Mora et al., 2009; Fu et al., 2018; Li et al., 2019b). Therefore, the effect of soil Cd on LMWOAs production in chicory and plantain, and the impact of plant produced LMWOAs on Cd uptake and translocation to aerial tissues of forage crops, was a key topic addressed in this study.

Following on from a review of existing literature, the following key research questions were identified:

(i) How does Cd in growth media effect on quantity and composition of LMWOAs secreted by chicory and plantain roots?

(ii) How does the root secrete LMWOAs influence plant Cd uptake and translocation by chicory and plantain?

(iii) Can a chemically modified carbon paste electrode be developed to measure the free Cd^{2+} ion concentration in the plant sap?

(iv) How does the concentration and production of LMWOAs in chicory and plantain xylem sap vary as a function of Cd in the growing media?

(v) How does xylem sap produce LMWOAs effect Cd translocation in chicory and plantain?

(vi) How do the variations in LMWOA root secretion and xylem sap LMWOA production in both plants explain the differences in plant Cd uptake by chicory and plantain?

(vii) How does the exogenous LMWOA impact on the shoot and root Cd concentration in chicory?

This final Chapter 7 presents an integrated discussion of findings over the individual research chapters of the thesis to summarise the key outcomes of the work.

7.2 Key Findings

7.2.1 The composition and quantities of LMWOAs in chicory and plantain root exudates and xylem sap vary as a function of Cd levels in growing media.

Two greenhouse experiments (Chapter 3 and 5) were established with different Cd concentrations in the growth media to evaluate the effect of Cd in growth media on LMWOA secretion by roots of chicory and plantain (Chapter 3) and production in xylem sap (Chapter 5). Chapter 3 explains that the composition and quantity of LMWOAs secreted by chicory and plantain roots varied as a function of the added Cd concentration in the growth media. Oxalic, fumaric, malic and acetic acids secreted by chicory; and oxalic, fumaric and malic acids secreted by plantain were the major LMWOAs analysed for all Cd treatments. Chicory showed lower secretion of fumaric acid and higher secretion of acetic acid compared to plantain. There was no clear difference observed

between oxalic and malic acid secretion between the two plants as a function of Cd in growth media. Similarly, Chapter 5 illustrated that both chicory and plantain showed variable production of oxalic, fumaric, citric, malic and acetic acids, in the xylem sap as a function of the Cd concentration in growth media. When the two species were compared, chicory produced more of all LMWOA (except fumaric acid) for all Cd treatment levels relative to plantain. Further, oxalic, fumaric and malic acids were common acids in root exudates and xylem saps of both plants.

7.2.2 A thiosalicylic acid modified carbon paste electrode developed in this thesis measured free Cd ions in environmental media.

A thiosalicylic acid modified carbon-paste electrode was developed as an alternative and reliable measurement tool for the detection of free Cd^{2+} ions in the environmental samples, including xylem saps. Thiosalicylic acid is a readily available commercial off-white solid, which is stable to air, and this makes it a conveniently handled ligand (Wehr-Candler and Henderson, 2016) to develop a Cd^{2+} ion-specific electrode with compared to other Cd^{2+} ion ligands used to develop Cd^{2+} ion specific electrodes in literature. The developed electrode showed a lower detection limit of 11 µg Cd/L (0.1×10^{-6} mol Cd/L) with a linear range from 20 to 100 µg Cd/L (0.18×10^{-6} to 0.88×10^{-6} mol Cd/L). To the best of my knowledge this is the first time a Cd^{2+} ion-specific electrode was developed to specifically determine free Cd^{2+} ion concentration in the plant xylem sap. The optimised parameters for electrode composition, type and pH of the supporting electrolyte and pre-concentration time for the modified electrode are presented in Table 7.1. The modified electrode measured free Cd^{2+} ion concentrations in a range of environmental media, including xylem saps with a high precision (RSD<5%). The electrode developed in Chapter 4, used to determine the free Cd^{2+} ion concentration in the

chicory and plantain xylem sap to understand the forms of Cd translocation in xylem sap (Chapter 5). The measurements of the electrode showed that the Cd in the xylem sap of chicory and plantain existed mainly in a complexed form than in free Cd^{2+} ion form.

Table 7.1. Parameters for Cd^{2+} ion detection using TSA-CP electrode.

Parameter	Optimised value
Electrode composition	15% (w/w) Thiosalicylic acid, 24% (w/w) paraffin oil, 61% (w/w) graphite powder
Supporting electrolyte	0.1 mol/L sodium acetate buffer
the pH of the supporting electrolyte	pH 4.5
Pre-concentration time	500s
Sample rate	5 Hz
Pulse amplitude	0.05 V

7.2.3 Low secretion of fumaric acid and high secretion of acetic acid by chicory roots and fumaric acid production in chicory xylem facilitate elevated shoot Cd accumulation in chicory compared to plantain.

The primary cause for the significant increase of shoot and root Cd concentration in both chicory and plantain, as a function of treatment level, was the significantly greater bioavailable Cd concentration in growth media with increasing Cd treatment level (Chapter 3 and Chapter 5). However, Chapter 3 shows a significantly higher shoot Cd accumulation in chicory (18.63 mg Cd/kg DW) than plantain (4.22 mg Cd/kg DW) at the highest tested soil Cd concentration (1.6 mg Cd/kg), while Chapter 5 showed a higher shoot Cd concentration compared to plantain for the same treatment at all Cd treatment level (except for control and 0.01 mg Cd/L treatment). This shows that different plants have different abilities to uptake Cd from roots and translocate to shoot. However, based on the findings of this thesis, this differential response between chicory and plantain can be explained by variations in the LMWOA concentration in root exudates and xylem sap for the two plants. The greater shoot Cd accumulation in chicory relative to plantain can now be explained by increased acetic acid and reduced fumaric acid secretion by chicory

compared to plantain (Chapter 3). This explanation was confirmed by the results of Chapter 6 where there was a reduction in shoot Cd concentration with increasing fumaric acid concentration in the hydroponic solution. This reduction was greatest at a concentration of 50 mg/L (50:1 treatment) fumaric acids in the growth media for shoots, and 10 mg/L (10:1 treatment) for roots. The external application of acetic acid to the growth media only increased shoot Cd concentration at a low added acetic acid treatment concentration (1 mg/L) (1:1 treatment), whereas for roots the maximum increase was at an external acetic acid concentration of 50mg/L (50:1 treatment). Free Cd^{2+} analysis of the xylem sap using the thiosalicylic acid-modified electrode showed Cd in the xylem sap of chicory and plantain existed dominantly in a complexed form (Chapter 4 and 5). Analysis of LMWOA in the xylem sap of chicory and plantain showed that variations in chicory shoot Cd and xylem sap Cd concentrations had a significant relationship with xylem sap LMWOA, while there was no relationship between shoot Cd concentration and xylem sap LMWOA in plantain. The functional relationships between chicory shoot Cd concentration and xylem sap LMWOA concentrations and hydroponic Cd concentration showed that hydroponic Cd concentration and xylem sap fumaric acid concentration are the dominant factors controlling shoot Cd accumulation in chicory. Statistical analysis showed that these factors explained 88% of the variability in chicory shoot Cd concentration observed in this thesis.

7.2.4 There is potential for LMWOA to reduce toxicity in chicory under pastoral farming conditions

Chicory grown in two hydroponic experiments showed a significant ($P<0.05$) reduction in biomass at higher Cd levels (>0.01 mg Cd/L treatment) (Chapter 5 and 6). Chapter 5 explains that LMWOAs in chicory xylem sap had an ameliorative effect on Cd toxicity

in plant tissues only at a low concentration of Cd in the growth media (i.e. the 0.01 mg Cd/L treatment).

Chapter 6 suggested that the limited Cd detoxifying ability of LMWOA could be a result of the reduction of stability and efficacy of the externally applied LMWOA overtime. The addition of increasing concentrations of fumaric, acetic and citric acids to the growth media in the presence of 1 mg Cd/L concentration did not show any strong ameliorative effect of LMWOA on Cd toxicity at any concentration of all three LMWOAs used in this study (Chapter 6).

However, a solution concentration of 1 mg Cd/L is excessively high in the context of New Zealand pastoral soils. The pot experiment in Chapter 5 showed that there was no significant growth reduction in chicory when grown in different native (different Cd concentrations) agricultural soil types (Chapter 5). The bioavailable Cd concentration in New Zealand agricultural soil is low reported to be less than 0.1 mg Cd/kg. Applying our findings of Chapter 5 stated above, the low bioavailable Cd concentration triggers a potential effect of LMWOAs to reduce the Cd toxicity in chicory when they grow in agricultural field conditions in New Zealand.

7.3 Importance of these findings for pastoral agricultural systems

Chicory and plantain are increasingly being grown in New Zealand pastoral soils as specialist summer and livestock finishing crops (Somasiri et al., 2015). Both plants are commonly sown as monoculture stands or in combination with legumes such as white and red clover. However, studies showed that chicory can accumulate high shoot Cd concentration from even low Cd soils to levels that might exceed regulatory guidelines for Cd in fodder crops and food compared to plantain. A field study done by Martin et al.

(1996) showed that even at extremely low soil total Cd concentrations (0.004-0.017 mg Cd/kg), chicory was able to accumulate high leaf Cd concentrations (1.6-2.4 mg Cd/kg DM) and this is of concern to food value chains. Of all the pasture species (including plantain) tested by Stafford et al. (2016), chicory showed the highest uptake concentration of Cd, and this study presents a possible mechanism to explain the elevated Cd uptake and translocation potential of chicory. Previous studies have reported differential secretion of fumaric and acetic acid by plant roots to explain the variation in shoot Cd accumulation in plants. For example, Cieśliński et al. (1998) reported that a high shoot Cd accumulating wheat cultivar (Kyle) showed low fumaric acid secretion and high acetic acid secretion compared to a low shoot Cd accumulating wheat cultivar (Arcola) when plants were grown in York soil. Adeniji et al. (2010) reported that the low shoot Cd- accumulating wheat cultivar (W9261-BG-L) had a higher fumaric acid concentration in roots compared to a high shoot Cd-accumulating cultivar (W9261-BG-H). As a strategy to potentially reduce Cd accumulation in chicory, it may be possible to upregulate the expression of genes that are responsible for the production of fumaric acid and down-regulate the expression of genes responsible for acetic acid in the root cells. Alternatively, selective breeding of cultivars that show differential excretion of acetic acid and fumaric acid and lower production of fumaric acid in the xylem sap may achieve the same goal. This strategy could effectively develop low Cd accumulating chicory cultivars.

Quantification of the free Cd^{2+} ion concentration in environmental solutions has been a constraint on analysis of Cd speciation in biological samples. The thiosalicylic acid modified electrode developed in this work will help screen xylem saps of different chicory and plantain varieties and enable investigation of how the free Cd^{2+} ion concentration varies between low and high Cd uptake plants varieties. The identification of varieties

with low free Cd^{2+} ion concentration could also potentially underpin the development of new cultivars of chicory and plantain with low Cd uptake and translocating ability that can be used in New Zealand agricultural systems. The adoption of the strategies described here will contribute to reducing the trophic transfer of Cd along the food chain for New Zealand pastoral agricultural systems.

7.4 Recommendations for future research

To extend the findings of this doctoral research, there are several ongoing knowledge gaps that justify further research, and these are summarised in this section.

(i) Experiments should be conducted to screen presently available chicory cultivars in New Zealand which show variation in acetic and fumaric acid root secretion.

(ii) Genetically modified experiments should be conducted to develop new breeding of chicory by regulating the necessary genes for the production of fumaric acid in root exudates and xylem sap, and acetic acid in root exudates, to reduce higher Cd accumulation in chicory.

(iii) Experiments should be conducted to investigate how the different soil parameters such as other metal ions in soil and soil micro-organisms impact plant root fumaric and acetic acid secretion and xylem sap fumaric acid production in chicory.

This thesis has provided a key point to defining the role of root secreted and xylem sap LMWOA on Cd accumulation in forage species. The findings of this research would underpin efforts to develop new traits of forage plant species that might mitigate the continuing risk of Cd transfer into grazing animals via consumption of Cd-rich forages

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Appendix 1

Table A1.1. Organic carbon content in productive soil in New Zealand based on National Soils Database.

New Zealand soil order	Total organic carbon content (g C/kg)
Allophanic	91
Brown	55
Gley	57
Granular	44
Melanic	46
Oxidic	79
Pallic	33
Podzol	44
Pumice	58
Recent	38
Semiarid	18
Ultc	43

Appendix 2

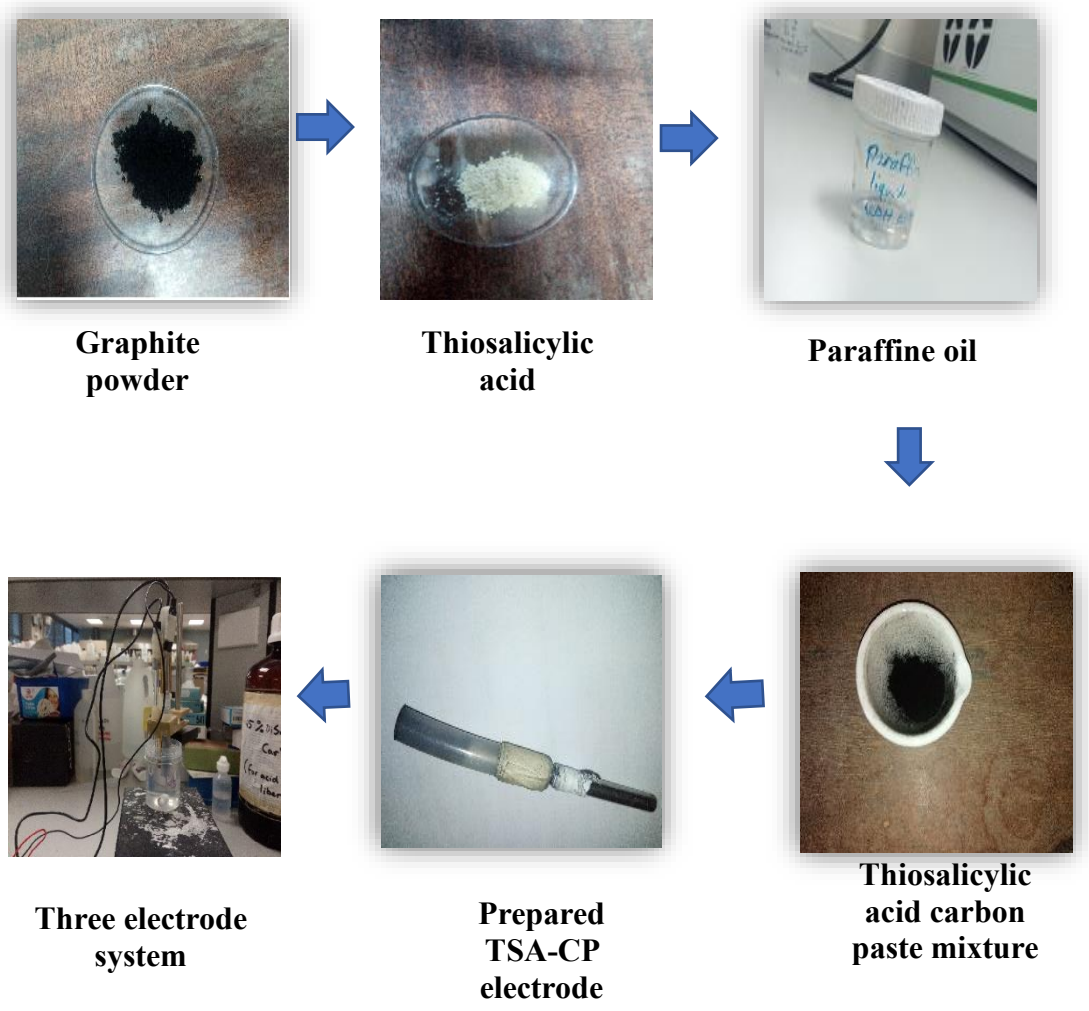

Figure A2.1. Graphical presentation of preparation of TSA-CP electrode.

Appendix 3

Table A3.1 Metal ion concentration in water samples.

Metal type	**Type of the water sample**		
	Tap water (µg/L)	**Drainage water (µg/L)**	**Wastewater (µg/L)**
Cu^{2+}	59.6	39.0	302.3
Pb^{2+}	56.0	18.6	445.1
Fe^{2+}	39.6	221.5	446.7
Al^{3+}	59.7	139.3	233.8
Mn^{2+}	320.1	240.3	654.5
Ca^{2+}	18700	12640	50000
Mg^{2+}	4020	4058	8029

Appendix 4

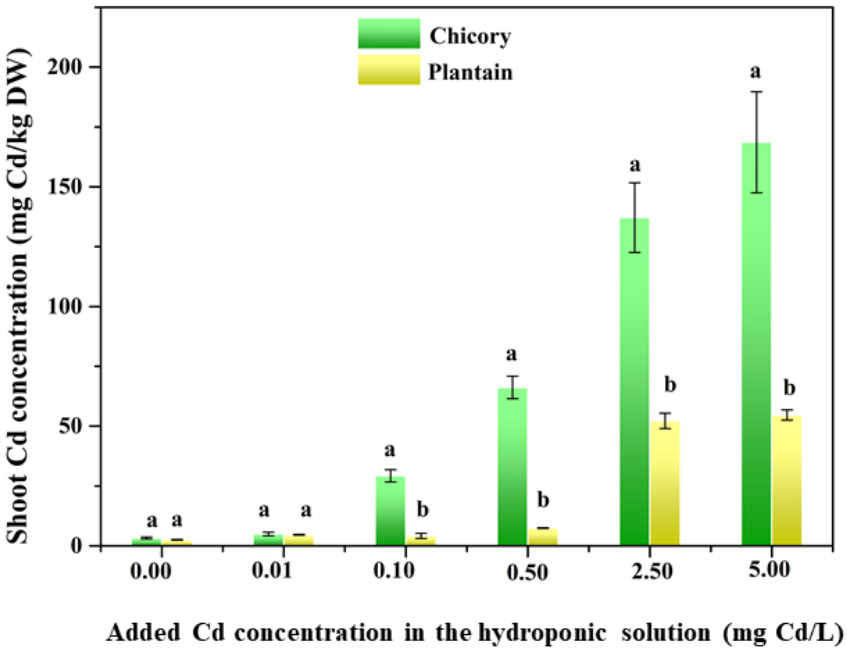

Figure A4.1. Significant differences of shoot Cd concentration between chicory and plantain with increasing Cd concentrations in the hydroponic solution. Significant differences shoot Cd concentrations between chicory and plantain are represented by (a-d) letters. Values in each bar followed by different letters are significantly different at $P<0.05$. Vertical error bars represent ±SE (n=3).

Appendix 5

Table A5.1. Summary of soil and plant Cd concentrations for plantain grown on three soil types.

Soil and Plant Cd concentrations (mg Cd/kg)	Soil types		
	Allophanic	Gley	Recent
Soil Total Cd	0.73±0.02a	0.43±0.02b	0.24±0.02c
Soil bioavailable Cd	0.24±0.01a	0.13±0.01b	0.02±0.00c
Plant root Cd	3.17±0.13a	1.50±0.09c	1.97±0.07b
Plant shoot Cd	2.51±0.30a	1.07±0.02b	0.55±0.04b
Xylem sap Cd[1] (mg/L)	0.01±0.00b	0.02±0.00b	0.08±0.01a

1 Measured from GFAAS

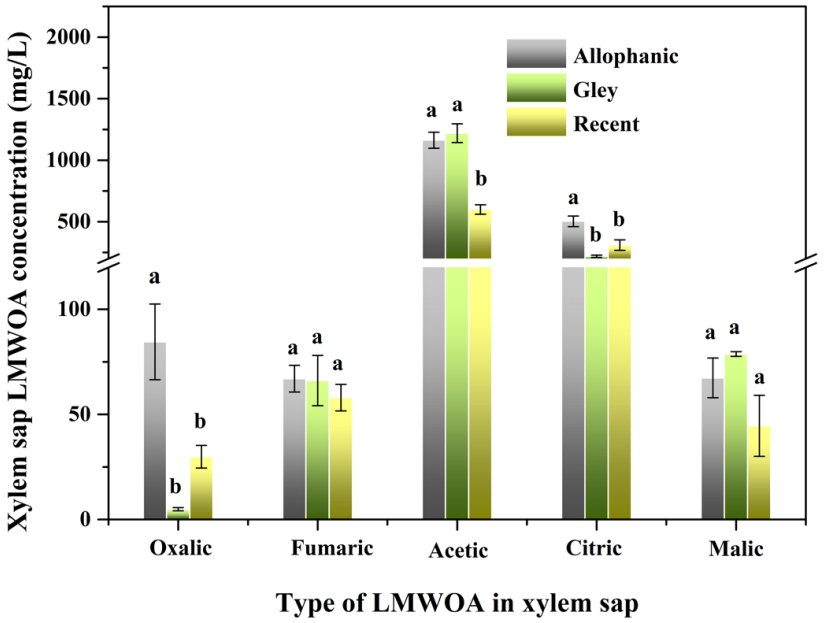

Figure A5.1. The significant difference of each LMWOA concentration of plantain between soil types are represented by a-d letters. Values in each bar, followed by different letters are significantly different at P<0.05. Vertical error bars represent ±SE (n=3).

Appendix 6

A6.1 Analysis of variation of solution pH and LMWOA with time

A small hydroponic experiment was set up in a greenhouse at the Massey University Plant Growth Unit with average day/night temperatures of 17/20 ℃. Growth media was a modified Hoagland solution adjusted to six increasing concentration ratios (mg/L) of LMWOA: Cd: 0:0 (control), 0:1(Cd-only), 1:1, 10:1, 50:1, and 100:1 with fumaric, acetic, and citric acid, independently. One health chicory plant was grown in each container. The composition of the hydroponic solution was same as the hydroponic solution used in Chapter 6. The pH of the solution was adjusted to 5.5-6.0 every day using 0.1M HNO_3 acid. The hydroponic solution in the containers (1L) did not renewed up to 7 days. The variation of the hydroponic solution pH was recorded every day. Further, 35 mL of the hydroponic solution was collected from each treatment everyday up to 7 days and the LMWOA concentration of that solution was analysed.

A6.1.1The hydroponic solution LMWOA analysis

The composition and concentration of LMWOAs in Hoagland solution were analysed by High-Performance Liquid Chromatography HPLC (Agilent Technologies 1200 Series, Santa Clara, CA, USA) as described by Cawthray (2003 with modifications. One mL of hydroponic solution was diluted with 2 mL of 25 mM KH_2PO_4 (the HPLC mobile phase solution). Each mixture was subsequently filtered through a 0.22 μm filter to remove suspended material prior to injection into the HPLC. Separation was conducted on a 250 × 4.6 mm (5 μm particle size) C_{18} reverse-phase column. Each sample solution (100 μL) was injected into the column with a flow rate of 1.0 mL/min at 25°C and UV detection at

210 nm. Potassium dihydrogen phosphate (25 mM) solution was used for isocratic elution. Identification of organic acids was performed by comparing retention times in samples with those retention times obtained by analysing a standard mixture including acetic, citric and fumaric acids.

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