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DIFFERENTIAL CADMIUM ACCUMULATION IN DURUM WHEAT: ROLE OF LOW MOLECULAR WEIGHT ORGANIC ACIDS

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DIFFERENTIAL CADMIUM ACCUMULATION IN DURUM WHEAT: ROLE OF LOW MOLECULAR WEIGHT ORGANIC ACIDS

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ABSTRACT

Pairs of isolines of durum wheat (*Triticum turgidum* var *durum*) differ in the amount of cadmium translocated to aboveground tissues; in the field, the high isolines have twice the cadmium in leaves and grain when compared to the low isolines. The hypothesis that differential cadmium translocation is associated with differential production of organic acids was tested by measuring cadmium in tissues, cadmium partitioning within the root, and organic acids in tissues and root exudates.

When grown in soil, no differences between high and low isolines were found. When grown in hydroponics, no differences in one pair (W9261-BG) were found. In the other pair (W9260-BC), the low isoline had half the cadmium in its shoot, increased cadmium in the root symplast and increased concentrations of fumaric, malic, oxalic and succinic acids compared to the high isoline. This suggests that reduced translocation to aboveground tissues was associated with increased chelation of cadmium in the root.

Keywords: Durum wheat, Cadmium, Isolines, Organic acids, Exudate, Symplast.

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CHAPTER ONE: INTRODUCTION

1.1. Cadmium and the Environment

Cadmium (Cd*) is a widespread toxic metal, and is rated to be the 5th most toxic metal to vertebrates and the 4th to vascular plants (Oberlunder and Roth, 1978). Owing to its toxicity, Cd^{2+} has been the focus of extensive research, and several studies have been done to investigate its effects on both plants and animals (reviewed in McLaughlin et al., 1999). The sources of Cd in the environment can either be natural from erosion of parent rocks (Heiny and Tate, 1997); or anthropogenic, including power stations, heating systems, metal-working industries, waste incinerators, urban traffic, cement factories and as a by-product of phosphate fertilizers (reviewed in Sanita di Toppi and Gabrielli, 1999).

The entry of toxic metals, including Cd, into agricultural soil poses a potential threat not only to crop plants but also to humans and animals consuming the harvested crops. Safety measures have been put in place to limit contamination of crops grown on agricultural soils. For example, agricultural soils are considered unsafe if the Cd concentration exceeds 1.4 mg Cd/kg (CCME, 2006). However, some agricultural soils have elevated Cd contamination. To illustrate, Frank et al. (1976) reported that the concentration of Cd in 296 soil samples from Ontario ranged from 0.1 to 8.1 mg/kg with a mean of 0.56 ± 0.69.

To meet the ever-increasing demand of our human population for food production, concerted efforts are being made to restore contaminated farm sites in order

* I have used Cd^{2+} to denote the cadmium-ion, which is water soluble, and 'Cd' to represent cadmium when the chemical forms are unknown. However, 'Cd' may include Cd^{2+}, cadmium chloride, cadmium-chelate, etc.

to reduce the high level of contaminants. Engineering-based efforts including physical and chemical removal of contaminated soils are costly and are now being substituted with a biological approach, phytoremediation (the use of green plants to clean-up contaminated sites), owing to its cost-effectiveness, environmental friendliness and ease of application, though it is a slow process (Ghosh and Singh, 2005).

1.2. Bioavailability and Uptake

1.2.1. Bioavailability of Cadmium in Soil

The maximum permissible concentration of Cd in agricultural soil of 1.4 mg Cd/kg soil (CCME, 2006) is based on the total Cd concentration in the soil. However, not all of Cd in the soil is available for uptake and transport; the fraction that is available for uptake is controlled by factors such as (1) cation exchange capacity (CEC) of the soil- metal cations bind with the exchangeable negative sites on the surfaces of soil particles (e.g., clay) thus decreasing metal bioavailability (Martinez and Motto, 2000); (2) pH- with a decrease in the soil pH, metal solubility and metal bioavailability increase (Basta et al., 1993); (3) soil organic matter- higher soil organic matter content increases the CEC and leads to increased Cd adsorption (reviewed in Grant and Sheppard, 2008); (4) competition from other cations e.g., zinc (Zn^{2+}) and addition of Zn^{2+} to Cd-contaminated soil resulted in the antagonistic interaction between the two cations with Zn^{2+} inhibiting the translocation of Cd^{2+} from root to shoot (Zhao et al., 2005); (5) biological processes (reviewed in Grant and Sheppard, 2008); e.g., arbuscular mychorrhizal can have dual

roles of either increasing or decreasing the bioavailability of Cd depending on the cultivars.

1.2.2. Nutrient Uptake by Higher Plants

Plants have root systems that perform essential adaptive functions including water and nutrient uptake, anchorage to the soil and the establishment of biotic interactions at the rhizosphere (Lopéz-Bucio et al., 2003). Upon reaching the root surface, mineral nutrients have two distinct routes for uptake and transport: (1) apoplastic route and (2) symplastic route (Clarkson, 1993) (see Figure 1.1)

(1) The cell wall and intercellular spaces comprise the apoplast, which is the pathway by which ions get into the cell. The apoplastic route is permeable to the transport of ions up to the endodermis where the presence of the Casparian Strip, an impermeable secondary thickening in the cell wall, restricts further transport (Tester and Leigh, 2000). Consequently, mineral ions following the apoplastic route are not available for transport via the stele.

(2) The cell membrane, cytoplasm and cell contents comprise the symplast. Living cells are interconnected by plasmodesmata, which allows for transport of ions, and other entities, from cell to cell. Once the ions have crossed the cell membrane, they are transported through the root symplast until they get to the stele.

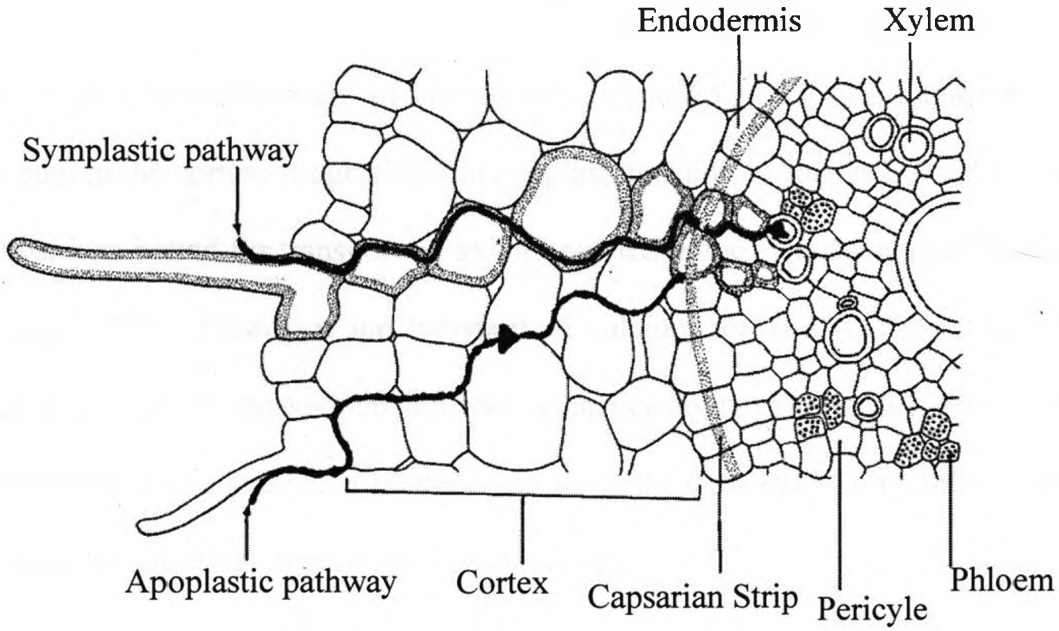


Figure 1.1: Symplastic and apoplastic pathways of ion absorption in the root-hair region. (adapted from Salisbury and Ross, 1985).

Regardless of the pathway across the root, ions destined for transport from root to shoot end up in the symplast and thus get to the conducting xylem cells. Both apoplastic and symplastic routes have been implicated in the uptake, transport and accumulation of toxic metals seeing that they follow same processes as the uptake of essential nutrients. For example, Casparian bands around the endodermal cells in *Hordeum vulgare* roots hindered the radial movement of lanthanum (La), and this led to the mass build-up of lanthanum in the cortex; suggesting that apoplast transport of ions is limited to the cortex and most ions bound for transport by xylem traveled in the symplastic route (reviewed in Clarkson, 1993). However, the transport of calcium ion (Ca^{2+}) in *Arabidopsis* roots (White et al., 2000) and sodium ion (Na^{2+}) in rice roots (Yeo et al., 1987) have been demonstrated to be unhindered by the presence of the Casparian Strip at the endodermis, suggesting an apoplastic bypass flow of these ions.

1.3. Cadmium Toxicity

In *Allium cepa*, Cd has been shown to damage the nucleoli of the root tips (Liu et al., 1995); in addition, Cd has been shown to alter the synthesis of RNA and inhibit ribonuclease activity in *Oryza sativa* (Shah and Dubey, 1995). Cd toxicity has also been linked to altered stomatal functions, the destruction of the photosynthetic apparatus and reduction in chlorophyll content (reviewed in Prasad 1995 and in Sanita di Toppi and Gabrielli, 1999), as well as leaf chlorosis, growth inhibition and water imbalance (Sandalio et al., 2001). Once in the root symplast, Cd has the potential to reduce the absorption and transport of nitrate by inhibiting nitrate reductase activity in the shoots

(Hernadenz et al., 1996). Cd has been implicated in the interference of the uptake and transport of other essential nutrients including calcium, magnesium, phosphorus and potassium (Das et al., 1997). Further, Cd toxicity to higher plants has been linked to increased production of free oxygen radicals or a decrease in enzymatic and non-enzymatic antioxidants (Balestrasse et al., 2001 and Cho and Sohn, 2004).

Cd toxicity is not limited to plants: it affects all living systems including animals. In Japan, consumption of rice contaminated with Cd led to loss of bone density, bone fracture and renal dysfunction in large numbers of people (reviewed in McLaughlin et al., 1999). The deleterious impact of Cd on humans has prompted most countries of the world to regulate the concentration of toxic metals in foodstuffs; e.g., the proposed maximum concentration for Cd in grains, as set by the Codex Alimentarius Commission, CAC (2005), is 0.2 mg Cd/kg.

1.4. Mechanisms of Metal Tolerance in Plants

Plants have homeostatic mechanisms that minimize the damage that non-essential metal ions can cause while keeping the concentrations of essential metal ions in cellular compartments stable (reviewed in Clemens, 2001). Some of the adaptive mechanisms employed by plants against toxic metals include immobilization, exclusion, chelation and compartmentalization, and each act by protecting sensitive tissues from toxic metals (reviewed in Cobbett, 2000 and Clemens, 2001). Chelators including phytochelatins (PCs) and low molecular weight organic acids (LMWOAs) have been implicated in

homeostatic regulation of toxic metals by forming metal-binding complexes with the toxic ions, and thus rendering them harmless.

1.4.1. Phytochelatins

Phytochelatins (PCs) are a family of small, metal-binding peptides with the general structure $(\gamma\text{-Glu-Cys})_n$ – X, where X could be alanine (Ala), cysteine (Cys), glutamine (Glu), glycine (Gly), or serine (Ser) and the value of n varies from 2 to 11 (Cobbett, 2001). They are induced by various metals but particularly by Cd (Cobbett, 2001 and Mirshra et al., 2006). PCs are synthesized from the tripeptide glutathione (GSH) and the reaction is catalyzed by the constitutively produced phytochelatin synthase (PCS) (Heiss et al., 2003 and reviewed in Benavides et al., 2005; Figure 1.2). After chelation of Cd^{2+} by PCs, the PC-Cd complexes are transported inside vacuoles through an ATP binding cassette (ABC) type transporter, where the PC-Cd complex dissociates and Cd^{2+} is then chelated with organic acids and PC is transported back into the cytosol to continue in its shuttling exercise (Ortiz et al., 1995 and reviewed in Sanita di Toppi and Gabrielli, 1999).

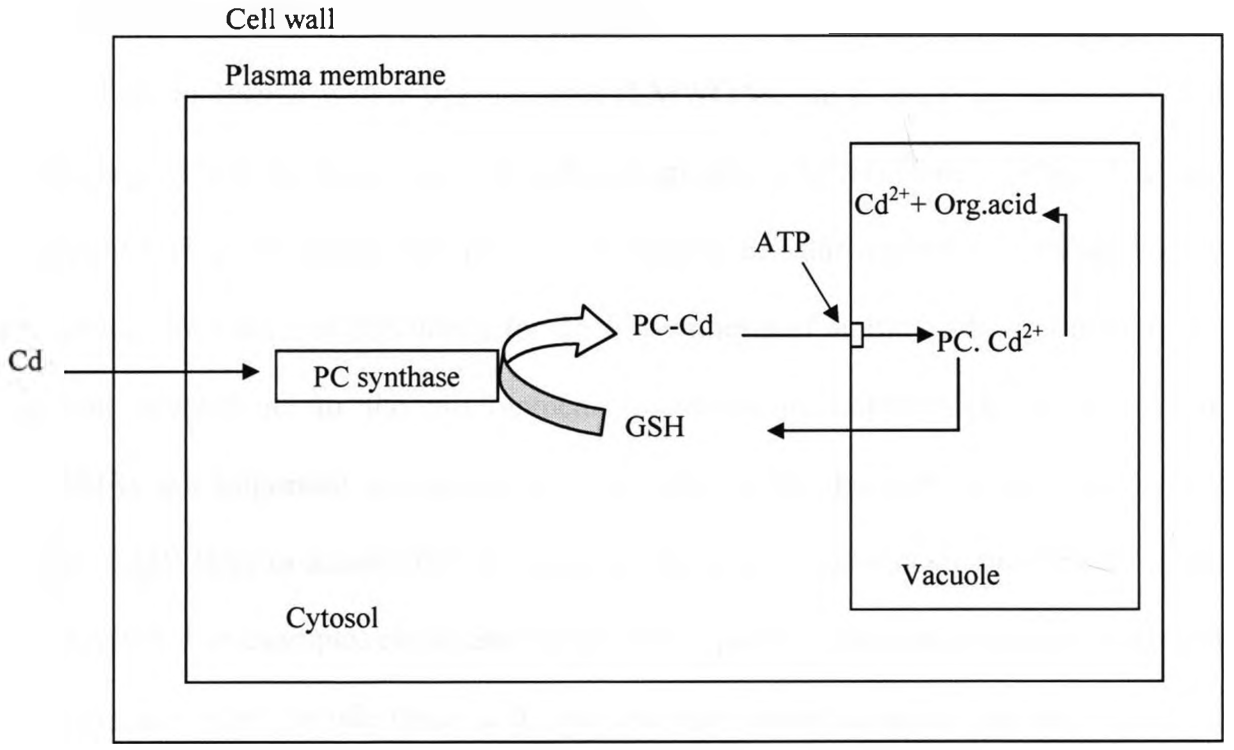


Figure 1.2: Schematic representation of the proposed mechanisms involved in Cd chelation and compartmentalization in the vacuole. PC-Cd (Phytochelatin-cadmium complex, GSH (Glutathione), ATP (Adenosine Triosphosphate), Cd^{2+} (Cadmium ion), Org. acid (Organic acids), (modified after Zenk, 1996 and Sanita di Toppi and Gabrielli, 1999).

1.4.2. Low Molecular Weight Organic Acids

Low molecular weight organic acids (LMWOAs) are found in all organisms, and are distinguishable by the presence of carboxyl groups (-COOH) (Jones, 1998). They are important biological compounds involved in several cellular reactions including energy production, formation of precursors for the biosynthesis of amino acids, and modulation of plant adaptations to the environment (reviewed in Lopéz-Bucio et al., 2000). LMWOAs are important compounds able to modify the rhizosphere, and plants may employ LMWOAs to access mineral nutrients that may be previously unavailable in the soil reserves. For example, citrate and malate are important complexers in calcareous soil (pH > 7), and plants exude these acids into the rhizosphere to induce the dissolution of previously unavailable insoluble Fe (Jones et al., 1996) and P (Jones, 1998; Lynch, 2007). LMWOAs are also important chelators in metal-tolerance, and have been involved in detoxification of metals owing to their negative charges which allow for the complexation of metal ions in solutions (Jones, 1998). Although produced in the mitochondria through the tricarboxylic acid (TCA) (Figure 1.3) or Kreb's Cycle, and in glyoxysomes as part of the glyoxylate cycle, excess LMWOAs are preferentially stored in the vacuole (Lopez-Bucio et al., 2000). As a mechanism of metal tolerance, organic acids are ubiquitous and have been widely reported in various plant species. They can be secreted extracellularly into the rhizosphere (apoplast) or transported intracellularly into vacuoles (Kochian, 1995 and Ma et al., 1997), where they can complex with metal ions.

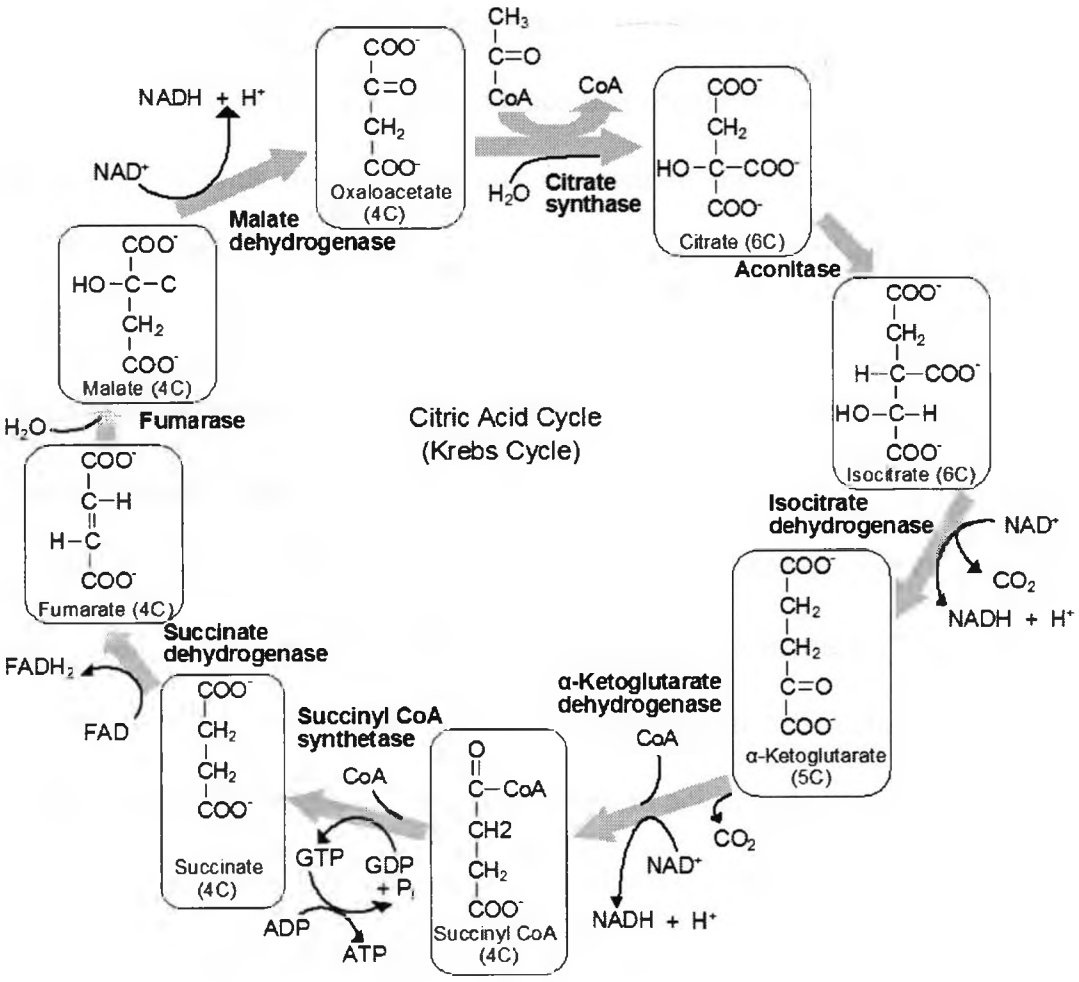


Figure 1.3: Schematic of the tricarboxylic acid (TCA) cycle. Redrawn from Russell et al. (2008).

1.4.2.1 Extracellular Organic Acids

Extracellular exudation of organic acids by plants is well documented in Al-tolerant species. There is unequivocal evidence that organic acids are secreted by several Al-tolerant species, thus making the roots resistant to the toxic effect of Al^{3+}. For example, in response to Al^{3+}, citric acid was secreted into the rhizosphere by *Hordeum vulgare* (Miyasaka et al., 1991), *Zea mays* (Pellet et al., 1995) and *Cassia tora* L. (Ma et al., 1997), while malic acid was predominantly present in the exudate from bread wheat (*Triticum aestivum* L.; Delhaize et al., 1993).

While endogenous organic acids in the rhizosphere seem to reduce metal toxicity, exogenous organic acids result in increased metal uptake. In their study, Nigam et al. (2000) reported that the concentration of Cd increased in Cd-treated wheat (*Triticum vulgare*) on addition of exogenous organic and amino acids, suggesting that organic acids increased the bioavailability and uptake of Cd. Further, organic acids including acetic, citric and malic acids added to hydroponically-grown barley (*Hordeum vulgare* L.) facilitated the root uptake of La and its subsequent accumulation in the shoot (Han et al., 2005). Similarly, Chiang et al. (2006) reported that organic acids added to soil played an important role in the solubilization and uptake of Cd by tobacco (*Nicotiana tabacum)* and sunflower (*Helianthus annuus*) grown in the greenhouse. While exogenous organic acids have been shown to increase the uptake of a number of metals by roots, data on the roles of endogenous organic acids exuded by plants are scarce and primarily focused on Al. It is imperative to know the relative contribution of endogenous organic acids in either conferring metal-tolerance to plants or in facilitating uptake of metals by roots.

1.4.2.2. Intracellular Organic Acids

While maintaining homeostasis, plants may deal with toxic metals in their tissues by complexation with organic acids, and subsequent sequestration in the vacuoles (reviewed in Sanita di Toppi and Gabrielli, 1999). Understanding the internal detoxification and complexation of toxic metals by organic acids has been achieved by studying metal hyperaccumulators – plant species that can accumulate approximately 100-fold higher concentrations of metal than general plant species (Prasad and Freitas, 2003). For example, Sarret et al. (2002) investigated the chemical forms of Zn in the Zn-tolerant hyperaccumulator *Arabidopsis halleri* compared with its non-tolerant, non-accumulator relative, *Arabidopsis lyrata* subsp. p*etraea*; in the aerial parts of *A. halleri,* Zn was predominantly complexed with malate. Similarly, Boominathan and Doran (2003) studied metal uptake and distribution in the hairy roots of a Cd-hyperaccumulator, *Thlaspi caerulescens,* and a Ni hyperaccumulator, *Alyssum bertolonii,* and found that about 13% of the total Cd in *T. caeruluscens* roots and 28% of the total Ni in *A. bertolonii* were complexed with organic acids. Also, Sun et al. (2006) in their study of a Cd-hyperaccumulator, *Solanum nigrum* L., showed accumulation of Cd to be positively correlated to acetic and citric acids in the leaves, suggesting these organic acids might be linked to its Cd-hyperaccumulation. It is therefore obvious that organic acids may play vital roles in metal tolerance, complexation and sequestration in the hyperaccumulators.

1.5. Durum Wheat

Durum wheat is one of the main staple foods consumed worldwide, and is one of several crops that tend to accumulate high concentrations of Cd when grown in Cd-

contaminated soil (Hart et al., 2006). Durum wheat is preferentially used in the food industries owing to several of its characteristics including hard grains, high grain density, high protein content and gluten strength (Bjorck et al., 1994). Among the major durum wheat-producing countries, Canada is on the frontline and it has been estimated that 3.8 million tonnes (Mt) of wheat grains were produced in the western prairie in 2007 (AAFC, 2007).

Health concerns associated with Cd-contaminated crops have caused the international food standard organization, the Codex Alimentarius Commission, to propose a 0.2 mg Cd/kg limit for wheat grains meant for the international market (CAC, 2005). This proposed limit may have dire consequences for durum wheat exporters from Canada where the natural accumulation of Cd in the grains varies from 0.1 – 0.5 mg Cd/kg depending on the cultivar (Garret et al., 1998).

1.5.1. Cadmium Accumulation in Durum Wheat

Recently, there has been intensive research on the five pairs of isolines of durum wheat (*Triticum turgidum* L. var *durum*) originally derived by Clarke et al. (1997; Table 1.1) in attempts to elucidate the mechanisms governing differential patterns of Cd uptake and accumulation, and to limit the Cd concentrations in durum wheat grain. These isolines were derived via classical breeding of different combinations of low and high Cd-accumulating cultivars: Kyle from the Swift Current breeding program; Nile from Aleppo, Syria; and others from either the Swift Current breeding program or Winnipeg.

Table 1.1: Five pairs of isolines of durum wheat derived by Clarke et al. (1997). The letters (L) and (H) behind the cultivar names represent low Cd- accumulators and high Cd-accumulators, respectively.

Cultivar Name	Genetic Stock	Derived from crossing
8982-SF (L)	GS-81,PI591058	Kyle/ Nile
8982-SF-(H)	GS-82,PI591059	
8982-TL-(L)	GS-83,PI591060	Kyle/Nile
8982-TL-(H)	GS-84,PI591061	
W9260-BC-(L)	GS-85,PI591062	DT61/DT471
W9260-BC-(H)	GS-86,PI591063	
W9261-BG-(L)	GS-87,PI591064	DT630/DT471
W9261-BG-(H)	GS-88,PI591065	
W9262-339A- (L)	GS-89,PI591066	Kyle/Biodur
W9262-339A- (H)	GS-90,PI591067	

Each pair of isolines can be distinguished into high and low Cd-accumulating isolines, with the high member of each pair having twice the Cd in their grains when compared to the low Cd-accumulating isoline (Archambault et al., 2001). The physiological basis for differential Cd accumulation in Clarke's five pairs of isolines remains evasive and unresolved, and will be the focus of this present work.

1.5.2. Differential Cadmium Accumulation in Isolines of Durum Wheat

Although many factors, including Cd uptake, xylem translocation from root to shoot, and production of metal-binding chelators have been studied to date, the observed differential Cd partitioning in these isolines has yet to be explained. Hart et al. (1998) investigated the uptake and xylem translocation of ^{109}Cd in durum wheat cultivars and reported that higher Cd accumulation in durum wheat (*Triticum turgidum* L. var *turgidum*) is unrelated to an increased rate of xylem translocation from roots to shoots but might be as a result of differential phloem loading. Also, Hart et al. (1998) observed that Cd binds to the cell wall in durum wheat and the concentration dependence of the binding was linear, suggesting that apoplastic binding might be responsible for the observed decreased in root to shoot Cd translocation. However, the relative involvement of the apoplast in differential Cd accumulation in the isolines of durum wheat remains unverified.

In another study, Harris and Taylor (2001) showed that the concentration of Cd in grains of the low Cd-accumulating isoline 8982-TL was 2-fold lower than in the high Cd-accumulating isoline, and the movement of Cd^{2+} into the grains of durum wheat treated with $^{109}Cd^{2+}$ was partly correlated with remobilization of Cd from the flag leaves. Further, Berkelaar and Hale (2000) examined the relationship between root morphology of six-day-old seedlings of Kyle (a high Cd-accumulator) and Arcola (a low Cd-accumulator) exposed to a range of Cd concentrations (approx. $4 \times 10^{-8} - 4 \times 10^{-7}$ M) in solution culture for 0 – 200 min and found that Kyle contained 35% less Cd per root system, 27.6% less root surface area and 21.2% fewer root tips after exposure to Cd. They suggested that the differential Cd accumulation in these cultivars could be linked to

differences in their root morphology. However, in their investigations on the uptake and translocation strategies employed by Kyle and Arcola, Chan and Hale (2004) speculated that the reduced accumulation of Cd in shoots of Arcola resulted from reduced root-to-shoot transfer of Cd at flowering and enhanced shoot-to-root retranslocation of Cd in younger plants. Similarly, Harris and Taylor (2004) investigated Cd uptake and translocation in the near-isogenic pair 8982-TL, and found no differences in the amount of Cd uptake by the roots of both isolines, but the rate of translocation of ^{109}Cd from roots to shoots was 1.8-fold higher in the high Cd-accumulating isoline, supporting their earlier report (Harris and Taylor, 2001) that restricted root-to-shoot Cd translocation may limit Cd accumulation in grains by directly controlling Cd translocation from roots during grain filling, or by controlling the size of shoot Cd pools that can be remobilized to the grain.

The roles of PCs and LMWOAs in differential Cd accumulation in *Triticum turgidum* L. (durum wheat) have also been studied. Stolt et al. (2003) investigated the involvement of phytochelatins in differential Cd accumulation in spring bread wheat (*Triticum aestivum*) and spring durum wheat (*Triticum turgidum* var *durum*), and found no differences in the distribution of PC chain lengths and PC isoforms, indicating that differential grain Cd accumulation did not correlate with differential PC-based Cd sequestration in the roots but might in part be due to differential Cd partitioning in the root. Thus, a closer look at the mechanisms of absorption and storage of Cd within roots might aid in elucidating the differential Cd concentrations in the isolines of durum wheat. Similarly, Bahrami (2006) found no correlation between concentrations PCs and Cd in the 5 pairs of isolines; all isolines produced PCs but their role in Cd accumulation was

unclear. Hart et al. (2006) investigated PC synthesis in the isolines of W9262-339A and found that soluble Cd in roots of both isolines was sequestered as a low-molecular-weight complex with PC, indicating that vacuolar influx of Cd is not different in the isolines. Perhaps differential efflux of Cd from vacuoles might regulate the translocation of Cd to the shoots.

Cieśliński et al. (1998) investigated the effects of LMWOAs exuded in the rhizosphere of durum wheat cultivars Arcola and Kyle. They found that Kyle exuded more LMWOAs to the rhizosphere when compared to Arcola, and suggested that this might be responsible for increased Cd uptake in Kyle. However, neither Arcola nor Kyle were selected on the basis of Cd accumulation, and it would be of great interest to know if Clarke's isolines, which were selected mainly on differential Cd accumulation in their grains, produce similar organic acid production patterns to those demonstrated by Kyle and Arcola.

1.6. Rationale and Objectives

Previous work in Sheila Macfie's laboratory has shown that two of the 5 pairs of isolines, 8982-SF and 8982-TL, did not differ in concentrations of LMWOAs in root and leaf tissues (McCutcheon, unpublished data; Sweeney, 2004). Further, Bahrami (2006) demonstrated that only within the W9260-BC and W9261-BG did the high isoline accumulate more Cd in the leaves than the low isoline when grown in hydroponic culture (with 0.1 μM Cd and after day 8). Thus, the current investigation focuses on differential Cd accumulation in these two pairs of isolines.

Since roots are the first site of exposure to Cd, the current study seeks to investigate the relationship between LMWOAs and Cd in the roots and in root exudates. Concentrations of organic acids in leaves will also be measured to assess the possibility of transportation of Cd-organic complexes in the plant. In addition, the relative contributions of internal (symplastic) versus external (apoplastic) immobilization of Cd in roots will be determined by measuring the relative amounts of Cd in the symplast and apoplast.

If LMWOAs play a crucial role in limiting Cd transport in durum wheat, I hypothesize that low and high Cd-accumulating isolines from near-isogenic pairs of *T. turgidum* var *durum* will produce different types and concentrations of LMWOAs when grown in the presence of Cd. As shown in the studies cited above, organic acids can have dual roles; they may either retain Cd within a tissue or facilitate transport of Cd between tissues.

To test this hypothesis, the following objectives will be carried out:

1. To measure the concentrations of Cd in root and shoot tissues of each isoline to verify differential patterns of Cd accumulation in the low and high Cd-accumulating isolines.
2. To determine the Cd partitioning in the apoplast and symplast of the two pairs of low and high Cd-accumulating isolines.
3. To identify and quantify the organic acids produced in roots and shoots in response to Cd.
4. To identify and quantify the organic acids exuded in response to Cd.

CHAPTER TWO: MATERIALS AND METHODS

2.1. Germination and Growth Conditions

For this study, two pairs of isolines of durum wheat (*Triticum turgidum* var *durum*) originally derived by Clarke et al. (1997) were used. The two pairs are designated as W9260-BC-L and W9260-BC-H, and W9261-BG-L and W9261-BG-H (L= low Cd-accumulating isoline, H = high Cd-accumulating isoline). The seeds were generously supplied by Dr. John Clarke (Agriculture and Agri-Food Canada, Swift Current, SK). The seeds were selected based on their differential Cd accumulation with the high Cd-accumulating isoline having twice the Cd in the leaves and grains than does the low Cd-accumulating isoline. Soil from the Brandon Research Station was provided by Dr. Cynthia Grant (Agriculture and Agri-Food Canada, Brandon, MB).

Seedlings were grown following a modified method based on Archambault et al. (2001). Briefly, seeds were pretreated in 1% sodium hypochlorite for 20 min, rinsed three times with distilled water, and thereafter imbibed overnight in aerated solution containing 0.005 g/L Vitavax (a systemic fungicide; Uniroyal Chemical Ltd, Calgary, AB, Canada). Aquaria were each filled with 10 L of S.I. nutrient solution (Table 2.1), adjusted to pH 6 with HCl, and the treated seeds placed on mesh suspended over the aerated nutrient solution. The aquaria were completely covered for 24 hours with black plastic to prevent seeds from drying out. Thereafter, the aquaria were uncovered and placed in a growth cabinet set to a day/ night temperature regime of 20/18°C with a 16 h light period and a 8 h dark period, and the fluorescent light intensity was 200 ± 15 μ mol. $m^{-2} \cdot s^{-2}$. Seedlings of uniform size were selected for one of two culture conditions: soil or hydroponics.

Table 2.1: Compositions of S.I. nutrient solution

Macronutrients	Concentrations (mM)	Micronutrients	Concentration (μM)
$Ca(NO_3)_2.\ 4H_2O$	1.0	H_3BO_3	6.0
K_2HPO_4	1.0	$MnCl_2.4H_2O$	2.0
KNO_3	0.4	$ZnSO_4.7H_2O$	0.5
$Mg(NO_3)_2.6H_2O$	0.3	$CuSO_4.5H_2O$	0.15
NH_4NO_3	0.3	Na_2MoO_4	0.1
K_2SO_4	0.1		
$FeCl_3.6H_2O$	0.01		
Na_2EDTA	0.01		

2.2. Soil Culture

Six-day-old seedlings grown in the aquaria were transferred into pots containing 110 g of prairie soil; one plant per pot with four replicates was grown in the growth cabinet under the same conditions as for germination (see section 2.1.). The plants were grown for three weeks and watered daily from the base.

2.2.1. Tissue Harvest and Preparation

Three-week- old plants were harvested and the roots were gently removed from the soil to minimize the damage to the roots, and desorbed in 20 ml of distilled water to

remove the rhizosphere soil. The roots were then blotted dry, separated from the shoot and fresh biomass recorded. The fresh tissues and the water containing the rhizosphere and its contents were processed for organic acid analysis (see section 2.5.1. and 2.5.2.). In parallel experiments, rinsed root and shoot samples were oven-dried at 60°C to constant weight and analyzed for Cd in the tissues (see section 2.4.).

2.3. Hydroponic Culture

On day 6, the seedlings were transferred into 1.4 L culture vessels (four plants per vessel and four replicates per treatment) filled with S.I. nutrient solution (pH 6), and aerated. Seedlings were placed between thin pieces of upholstery foam and suspended in slits cut into the lids of the culture vessels. The culture vessels were covered with black cloth to limit algal contamination, and placed in the growth cabinet (same conditions as for germination). The nutrient solutions were replenished every other day to ensure constant supply of nutrients and to safeguard against nutrient deficiencies. The solutions in culture vessels were brought up to 1.4 L daily to account for water loss due to evapotranspiration.

2.3.1. Experimental Treatment

Eight-day-old plants were treated with two different concentrations of $CdCl_2 \cdot 4H_2O$ (0 μM and 0.1 μM) added to fresh S.I. nutrient solutions. The nutrient solutions with or without Cd^{2+} supplementation were replenished every other day and aerated. The

seedlings were harvested after eight days of treatment. One bioassay for early identification of low Cd-accumulating isolines of durum wheat was developed by Archambault et al. (2001). They observed a significant increase in Cd accumulation in the second leaf of high Cd-accumulating isoline exposed to Cd for eight days when compared to the low Cd-accumulating isoline. Thus, this rapid, seedling-based bioassay allows for identification of low and high Cd-accumulating isoline without having to grow the plants until maturity.

2.3.2. Tissue Harvest and Preparations

Plant tissues were harvested on day 8 after treatment with or without Cd^{2+}, and fresh weight was recorded. To measure the amount of Cd^{2+} in the symplast, half of the Cd-treated roots of each isoline and their corresponding controls were rinsed in dH_2O for 30 s followed by a 30 min wash in 1 mM $CaSO_4$ and another 30 s wash in dH_2O (Taylor et al., 1998); this is to remove any extracellular (apoplastic) Cd by cation exchange reaction between Cd^{2+} and Ca^{2+}.

Similarly, the other half of Cd-treated roots and their respective controls were washed only in dH_2O for 30 s to rinse the nutrient solution and then processed to measure the total Cd^{2+} in the root tissues. In either case, the plant tissues were blotted dry with tissue paper, separated into roots and shoots and oven-dried at 60°C to constant weight and analyzed for Cd^{2+}. With these treatments, the amount of Cd in the apoplast can be obtained by subtracting the amount of Cd^{2+} in the symplast from the total Cd^{2+} in the roots (section 2.4.).

2.4. Measurement of Cadmium

Dried plant tissues (grown hydroponically or in soil) were weighed, cut into pieces and approximately 0.1 – 0.2 g were weighed into individual test tubes. Similarly, 1.0 g of dried prairie soil (three replicates) was analyzed for total Cd^{2+}. To the individual samples, 1.5 ml nitric acid (Omni-Trace®) was added. Each test tube was capped with a marble to keep the sample in the tube and allow for pressure release. Racks of test tubes were allowed to sit at room temperature overnight, then placed on a sand-filled tray and heated to 90-100°C until the tissues were fully digested. Tomato leaves (NIST standard # 1573a) and reagent blanks were included in the digestion process to allow for easy determination of percentage recovery of Cd^{2+} in the tissue sample and possible contamination, respectively. To illustrate, the Cd^{2+} content of NIST tomato leaves is 1.52 µg/g and one would expect to detect this concentration if all tissue digestion were complete. Percentage recovery ranged from 82- 97%. The reagent blanks were expected to lack Cd. When cooled to room temperature, the samples were filtered using 9 cm VWR brand filter paper (qualitative 413) and the volume brought up to 25 ml with dH_2O.

The samples were analyzed for Cd^{2+} by inductively coupled plasma- atomic emission spectroscopy (ICP-AES) using a Perkin-Elmer Optima 3300 dual view ICP-AES, with a RF generator power of- 1300 Watts, gas flow rate- 15 L· min^{-1}, auxiliary flow rate- of 0.5 L· min^{-1}, nebulizer flow rate- of 0.8 L· min^{-1}, pump (for sample) flow rate- of 1.0 L· min^{-1}, and an Analyte line- of Cd 226.507 nm, with a detection limit of 8-10 ppb for Cd.

2.5. Organic Acid Analyses

2.5.1. Organic Acids in the Exudates

For plants grown in the prairie soil, the roots were gently removed from the soil in order not to minimize damage to the root cells, and were rinsed in 20 ml distilled water to remove the soil adhering to the root surface (rhizosphere). This technique did not permit measurement of rhizosphere/soil mass. The soil-water mixture was placed on a mechanical shaker for 1 h at 125 rpm and 1 ml of the supernatant was transferred into an Eppendorf tube and centrifuged at 13,000 x g for 10 min at 4°C. For plants grown in the hydroponic culture medium, 10 ml of the spent medium was frozen and stored (-20°C) in centrifugation tubes until analyzed. For organic acid analysis of the exudates from either soil-cultivated or hydroponically grown plants, 300 µl each was injected into the ion chromatography (IC, Dionex; section 2.6.1.) without further preparation. Organic acids in the exudates solution were quantified in mM in either case because the soil on the root surface had been previously desorbed in distilled water.

2.5.2. Organic Acids in Plant Tissues

Plant tissues were carefully monitored during growth and development to ensure adequate nutrient supply and to raise the confidence in organic acid data because studies have shown that organic acids may be produced by nutrient-deficient plants as in the case of lupin, *Lupinus albus* L. cultivars Minori and Nelly, where exudation rate for citric acid increased significantly under phosphorus deficiency (Egle et al., 2003).

Following harvest, plant tissues (roots and shoots) grown in soil or hydroponic culture were prepared using the modified method of Sanita di Toppi et al. (2007). The roots were severed from the shoot and fresh tissues (approximately 1.0 g) were ground to powder in a chilled mortar using liquid nitrogen, collected in 50 ml centrifuge tubes and frozen (-80°C) until analysis. For analysis, 5 ml of distilled water was added to the tissue samples and the mixture was heated for 20 min in a water bath at 80°C to denature the degradative enzymes. The mixture was then centrifuged at 2500 xg for 10 min at 25°C and 300µl of the supernatant was injected directly on an IC, Dionex system for the determination of organic acids. Where appropriate, the supernatant was diluted to reduce overloaded peaks and to have more resolved and identifiable peaks.

2.6. Ion Chromatography

2.6.1. Organic Acid Analyses Using Ion Chromatography

The method used for the separation of organic acids using Ion Chromatography (IC) followed that of Liu et al. (2007) with slight modifications. Briefly, the organic acids were separated with a Dionex ICE-ASI Ion exclusion column (4 x 250 mm) with the following conditions: column settings for the Dionex- 100 system included 1.5 mM heptafluorobutyric acid (HFBA) as eluent, eluent flow -80 on the dial, nitrogen pressure – 7.5 psi, run time 11.90 min, 40 µl injection volume. The suppressor settings for the Dionex- 100 system were: regenerant- 9.7 mM tetrabutyl ammonium hydroxide (TBAOH), regenerant flow- 3.5 ml/min and nitrogen pressure- 15 psi.

Organic acid standard mixtures for the major organic acids including citric, fumaric, malic, malonic, oxalic and succinic acids (Sigma, St. Louis, Missouri) were made in distilled water, assayed using the IC (Figure A1) and the calibration curves for individual organic acids were determined from the areas under the peaks corresponding to different concentrations (Figure A2).

2.6.2. Peak Identification

Spiking experiments involving the addition of known concentrations of organic acid standards to the supernatants were carried out in order to validate the identities of the observed peaks.

2.7. Statistical Analyses

All statistical analyses were performed using SigmaStat Version 2.0 and MS-Excel. For each sample set, the mean, standard deviation and standard errors of mean (SEM) were calculated using MS-Excel for Windows. A two-way analysis of variance (ANOVA) was used to determine the effects due to species and Cd treatment and where no interactions existed, Tukey test was used to determine significant difference between means ($P < 0.05$). Student's t-test was used to determine differences in mean values of total organic acids and Cd in the symplast of low and high Cd-accumulating isolines. Due to significant interactions between isolines and organic acids, one-way ANOVAs were used to analyze the isolines, and Tukey test was used to determine significant

differences between treatments. Exudates from soil-potted plants were analyzed with one-way ANOVA while repeated measures ANOVA was performed to determine the effects of Cd and time on organic acid exuded by plants grown in hydroponic culture systems. Graphs were produced using SigmaPlot Version 10.0 for Windows.

CHAPTER THREE: RESULTS AND DISCUSSION

The production of organic acids in response to Cd was studied in durum wheat grown in two culture systems: pots containing prairie soil, which had elevated concentrations of Cd, and in hydroponic culture with 0.1 μM $CdCl_2 \cdot 4H_2O$. These experiments were designed to determine the role of organic acids in differential Cd accumulation in the low and high Cd-accumulating isolines of durum wheat. Plants were monitored during growth and development to confirm that they did not show symptoms of Cd stress.

3.1. Soil Culture Experiments

3.1.1. Effect of Cd on Plant Biomass

Total Cd in the prairie soil was 17.57 ± 1.18 mg/kg but the DTPA-extractable Cd in the soil was only 0.17 ± 0.02 mg/kg. The dry weights for the two pairs of isolines of durum wheat are presented in Figure 3.1. With the exception of the low Cd-accumulating isoline of W9260-BC, which was heavier ($P = 0.034$) than the high Cd-accumulating isoline, the shoot and root dry weights within a pair did not differ ($P > 0.05$). Throughout the experimental period, the plants were healthy and showed no sign of Cd-induced stress or of nutrient deficiency (Figure 3.2). Stolt et al. (2003) also found no significant differences in the biomasses of durum and bread wheat cultivars grown in a range of Cd concentrations.

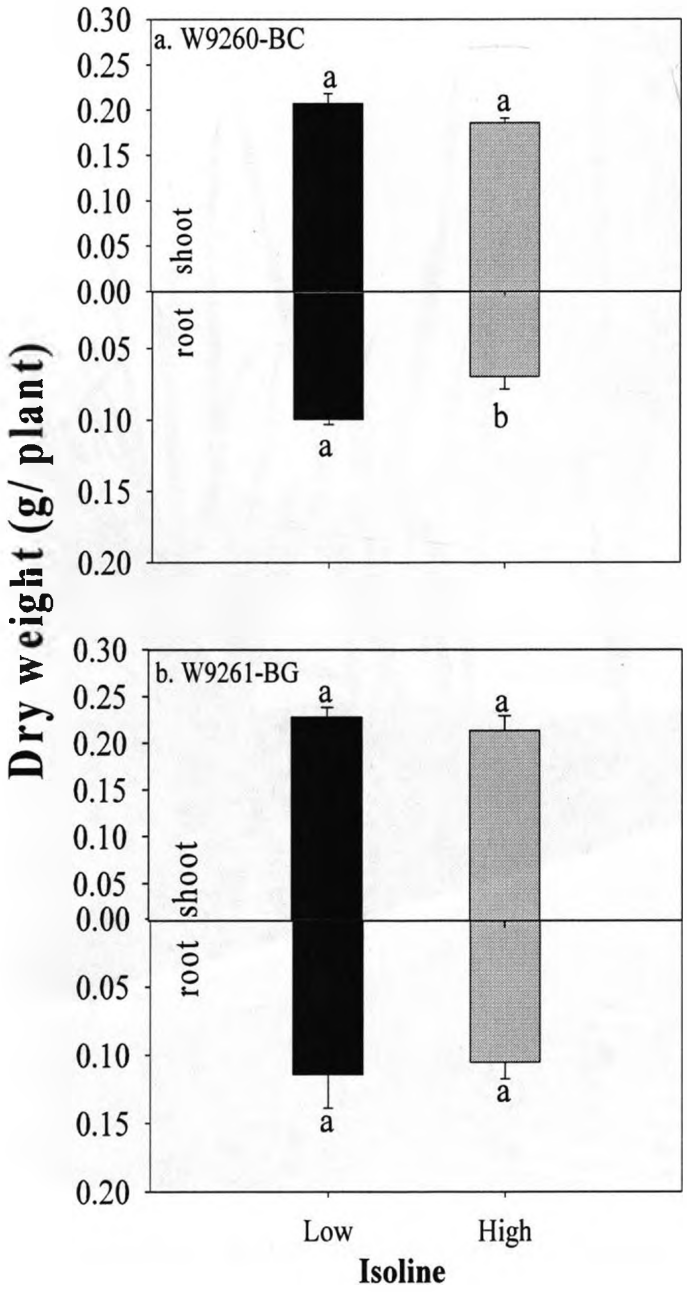


Figure 3.1: Root and shoot dry weights of low and high Cd-accumulating isolines of durum wheat grown in soil. Low and high Cd-accumulating isolines (a) W9260-BC and (b) W9261-BG were grown in soil for 21 d, harvested and oven-dried to constant weights. Different lower case letters denote significant difference ($P < 0.05$) between the low and high Cd-accumulating isolines. Error bars represent SEM of three replicates.

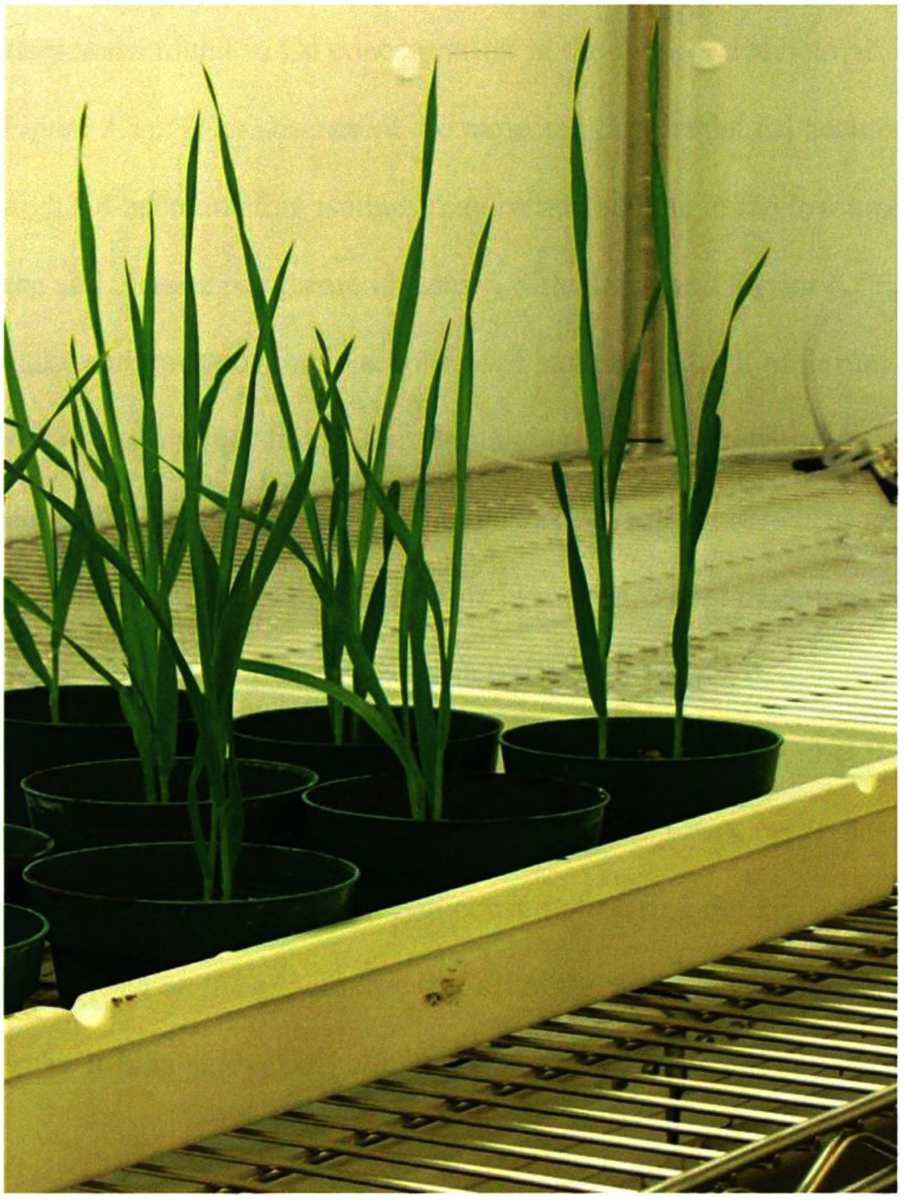


Figure 3.2: Experimental set up for the soil-cultivated plants. No signs of Cd-induced stress o nutrient deficiency were noticed throughout the experimental period.

3.1.2. Cadmium Accumulation in Plant Tissue

No differences were found in Cd concentration in the roots and shoots of the two pairs of isolines (Figure 3.3). The absence of the expected differential Cd accumulation between low and high Cd-accumulating isolines may be attributable to the low amount of extractable Cd in the soil. These results are in accord with Harris and Taylor (2004) who investigated Cd uptake and translocation in seedlings of isoline 8982-TL of durum wheat. They found that neither the low nor high Cd-accumulating isoline in their study differed in root Cd or uptake of Cd by whole plants. Similar results were reported by Bahrami (2006) who found no difference in Cd concentrations in root and shoot of isoline W9262-399A grown in nutrient solutions supplemented with 0.05 and 0.1 μM Cd.

Although the concentration of total Cd in the soil was 12-13 times higher than that permitted for agricultural soils (1.4 mg/kg; CCME, 2006), the plants were not highly contaminated; they were near the limit for human consumption (0.2 mg/kg; CAC, 2005). The CCME sets limits based on the assumption that all of the metal in the soil is taken up by the plant. As seen in this study, only the extractable Cd in the soil appeared to be available to plant.

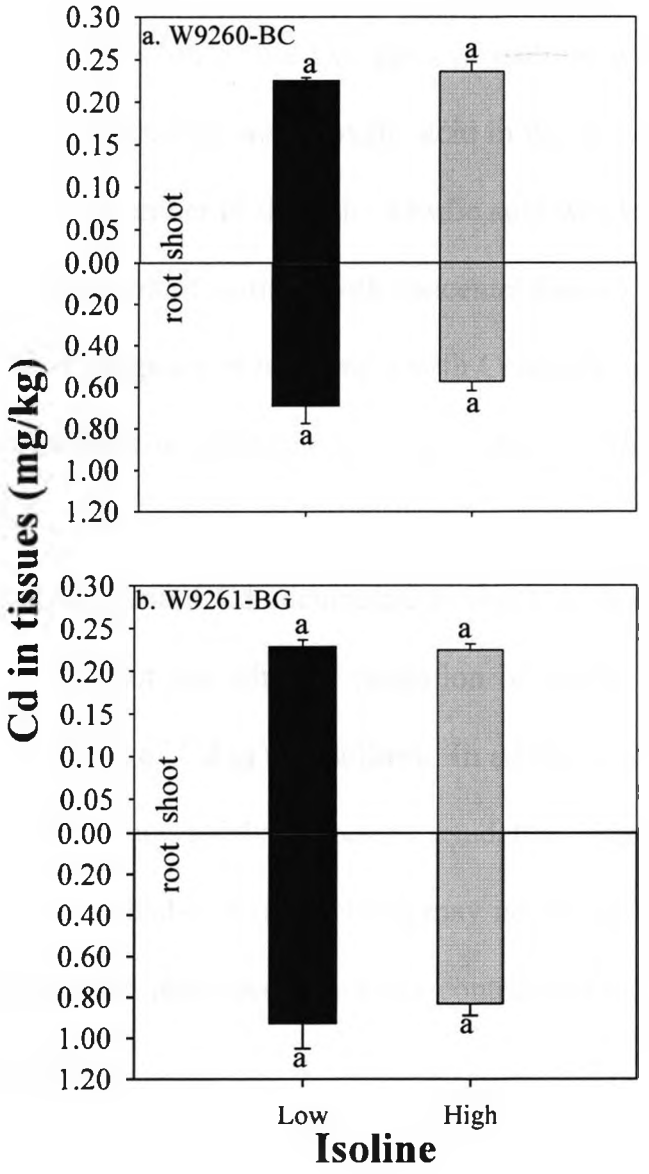


Figure 3.3: Concentrations of Cd in root and shoot of low and high Cd-accumulating isolines of durum wheat grown in soil. Low and high Cd-accumulating isolines (a) W9260-BC and (b) W9261-BG were grown in soil for 21 d, harvested and oven-dried to constant weights. Tissues were analyzed for Cd using ICP-AES. Same lower case letters indicate no difference between isolines ($P > 0.05$). Error bars denote SEM of three replicates.

3.1.3. Organic Acids in the Rhizosphere Soil

The rhizosphere soil of the two pairs of isolines was analyzed for organic acids (Figure 3.4). The concentrations of oxalic acid in the rhizosphere averaged between 0.8 and 1.0 mM in each member of the pair. Oxalic acid was found to be predominant in the soil exudate of both pairs of isolines with concentrations of citric and malic acids in trace amounts. These findings are in agreement with Cieslinski et al. (1997) who found oxalic acid to be predominant in exudates of durum wheat cultivars DT627, DT637 and flax cultivar YSED 2.

Because differential Cd accumulation was not observed in plants grown in soil (Figure 3.2), one cannot say whether exudation of oxalic acid is related to differential uptake or translocation of Cd in the isolines. In addition, the values for organic acids in the rhizosphere may not solely represent exudation from plant roots. The relative involvements of microbial-root interactions may not be completely ruled out in the soil-potted experiments and microbes may have contributed to the organic acid pool in the rhizosphere samples.

3.1.4. Organic Acid Content in Durum Wheat Grown in Soil

The results for the total organic acid content in the isolines are presented in Figure 3.5. Overall, in W9260-BC, the root and shoot did not vary in the total organic acid concentrations while the root of the high Cd-accumulating isoline W9261-BG had greater organic acids compared to the low Cd-accumulating isoline ($P < 0.05$) but no difference was observed in the total organic acids in its shoot. A closer look at individual organic acid components showed that four organic acids including citric, malic, oxalic and

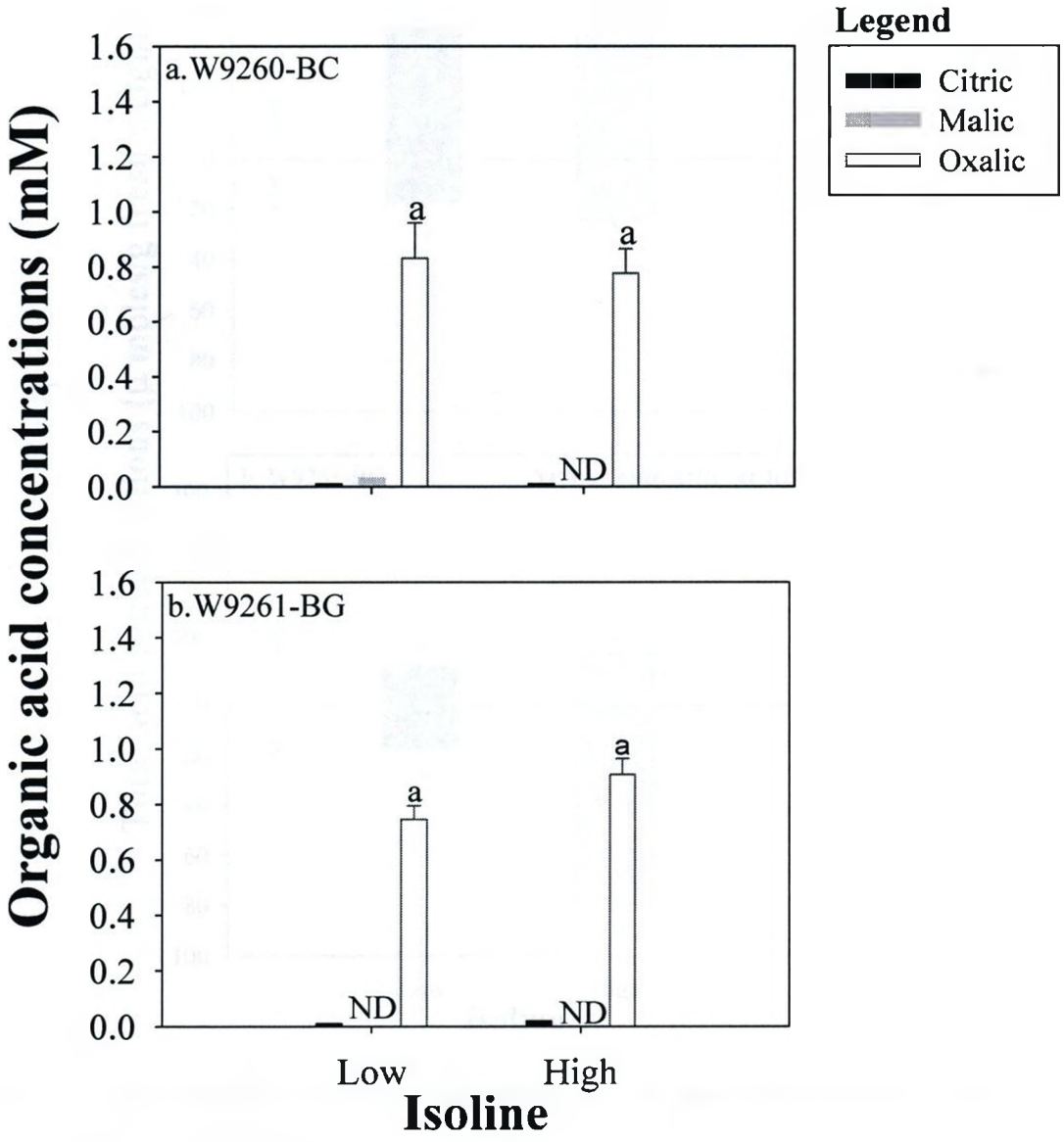


Figure 3.4: Concentrations of organic acids in the exudate of isolines of durum wheat grown in soil. Low and high Cd-accumulating isolines (a) W9260-BC and (b) W9261-BG were grown in soil for 21 d, harvested and the roots gently desorbed in distilled water to remove the rhizosphere soil. The solution was analyzed for organic acids using ion chromatography. Same lower case letters show no difference between isolines ($P > 0.05$). ND means that the organic acid was not detected or below detection limit for the IC. Error bars denote SEM of four replicates.

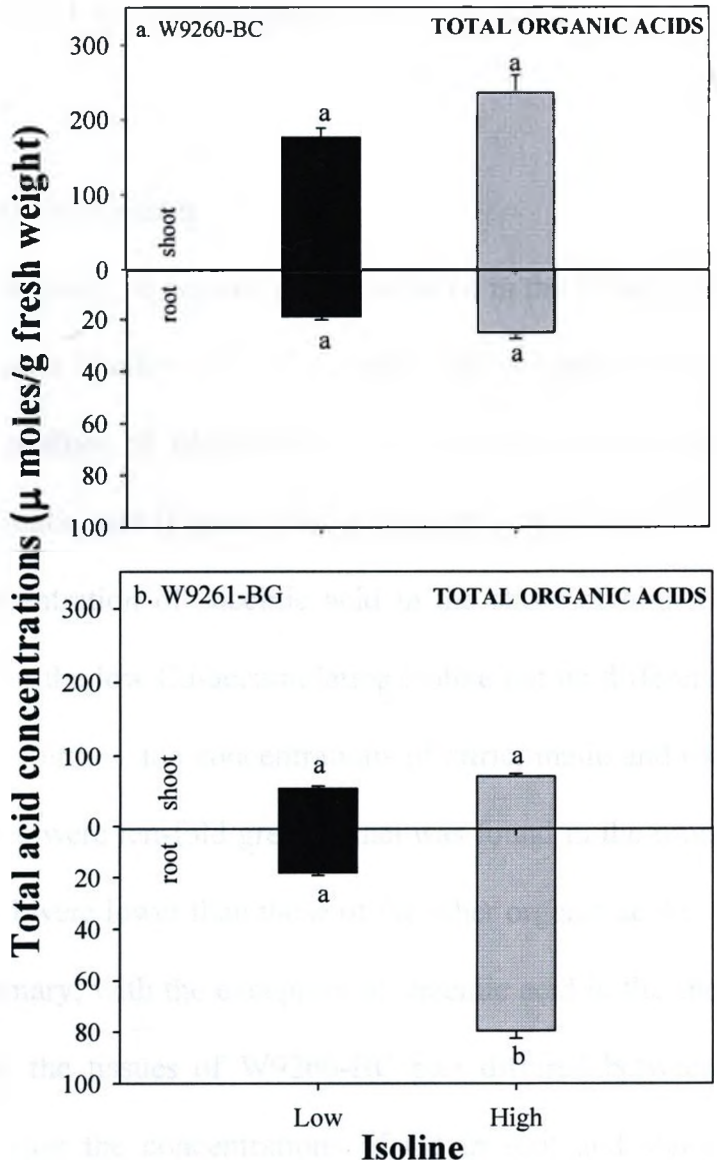


Figure 3.5: Total organic acid concentrations in root and shoot tissues of isolines of durum wheat grown in soil. Low and high Cd-accumulating isolines of (a) W9260-BC and (b) W9261-BG were grown in soil for 21 d, harvested ant tissues analyzed for organic acid using ion chromatography. Different lower case letters show significant difference ($P < 0.05$) between isolines. Error bars denote SEM of four replicates.

succinic acids were found in the W9260-BC pair while an additional acid, fumaric, was found in the W9261-BG (See below).

3.1.4.1. W9260-BC Isolines

Concentrations of organic acids produced in the tissues were in the order of oxalic acid > malic acid > citric acid > succinic acid (Figure 3.6). The low and high Cd-accumulating isolines of W9260-BC did not differ in the concentrations of citric acid (Figure 3.6a), malic acid (Figure 3.6b) and oxalic acid (Figure 3.6c) in the root and shoot. However, concentration of succinic acid in the shoot of high Cd-accumulating isoline was greater than the low Cd-accumulating isoline but no difference was found in its root (Figure 3.6d). Further, the concentrations of citric, malic and oxalic acids in the shoots for both isolines were ten-fold greater than was found in the roots but the concentrations of succinic acid were lower than those of the other organic acids.

In summary, with the exception of succinic acid in the shoot, none of the organic acids found in the tissues of W9260-BC pair differed between isolines. This is not surprising because the concentrations of Cd in root and shoot tissues did not differ between the isolines.

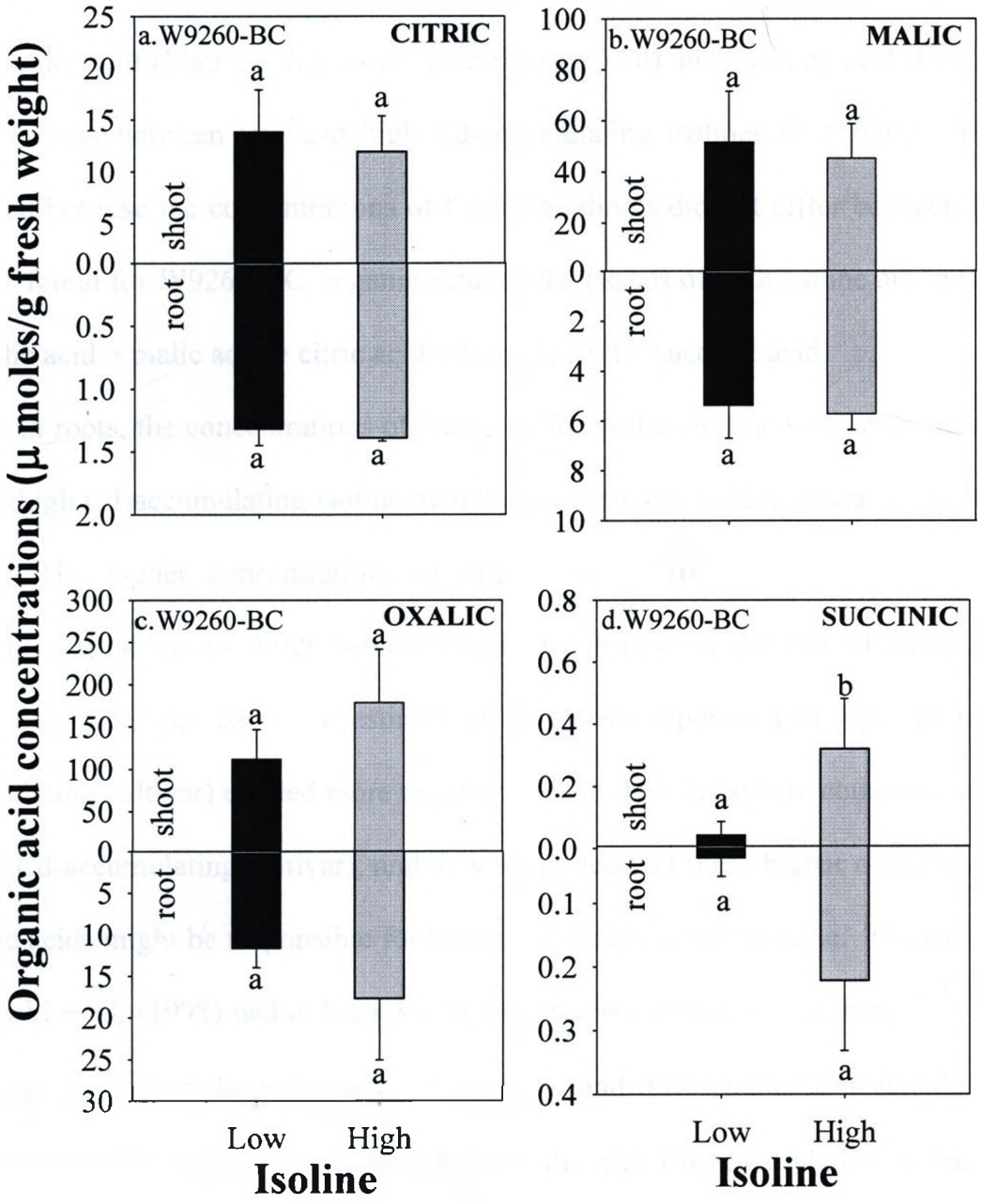


Figure 3.6: Concentrations of different organic acids in root and shoot tissues of W9260-BG grown in soil. Concentrations of (a) citric acid (b) malic acid (c) oxalic acid and (d) succinic acid in the root and shoot tissues were determined by ion chromatography. Different lower case letters between isolines show significant difference ($P < 0.05$). Error bars represent SEM of four replicates.

3.1.4.2. W9261-BG Isolines

In shoots, the concentrations of citric acid (Figure 3.7a), fumaric acid (Figure 3.7b), malic acid (Figure 3.7c), oxalic acid (Figure 3.7d) and succinic acid (Figure 3.7e) did not differ between low and high Cd-accumulating isolines ($P > 0.05$). This was expected because the concentrations of Cd in the shoots did not differ between isolines. As was found for W9260-BC, organic acids in the tissues of both isoline are in the order of oxalic acid > malic acid > citric acid > fumaric acid > succinic acid.

In roots, the concentrations of citric, malic, oxalic and succinic acids were higher for the high Cd-accumulating isoline than those of the low Cd-accumulating isoline ($P < 0.05$). The higher concentrations of organic acids in the roots of the high Cd-accumulating isoline of W9261-BG is interesting in light of the lack of differential Cd accumulation in this pair. Cieśliński et al. (1998) reported that Kyle (a high Cd-accumulating cultivar) exuded more organic acids in the rhizosphere compared to Arcola (a low Cd-accumulating cultivar), and they suggested that these higher concentrations of organic acids might be responsible for higher Cd accumulation in Kyle. The soil used by Cieslinski et al. (1998) had at least 3-fold higher concentrations of extractable Cd (6.3 – 191.4 $\mu g \cdot kg^{-1}$) than the soil used in this study, and differential Cd-accumulation was observed for their plants. In the current study, the high Cd-accumulating isoline did not take up more of the very low amounts of extractable Cd in the soil, but the increased production of organic acids in the high Cd-accumulating isoline may be a constitutive feature that allows the isoline to take in more Cd when it is bioavailable.

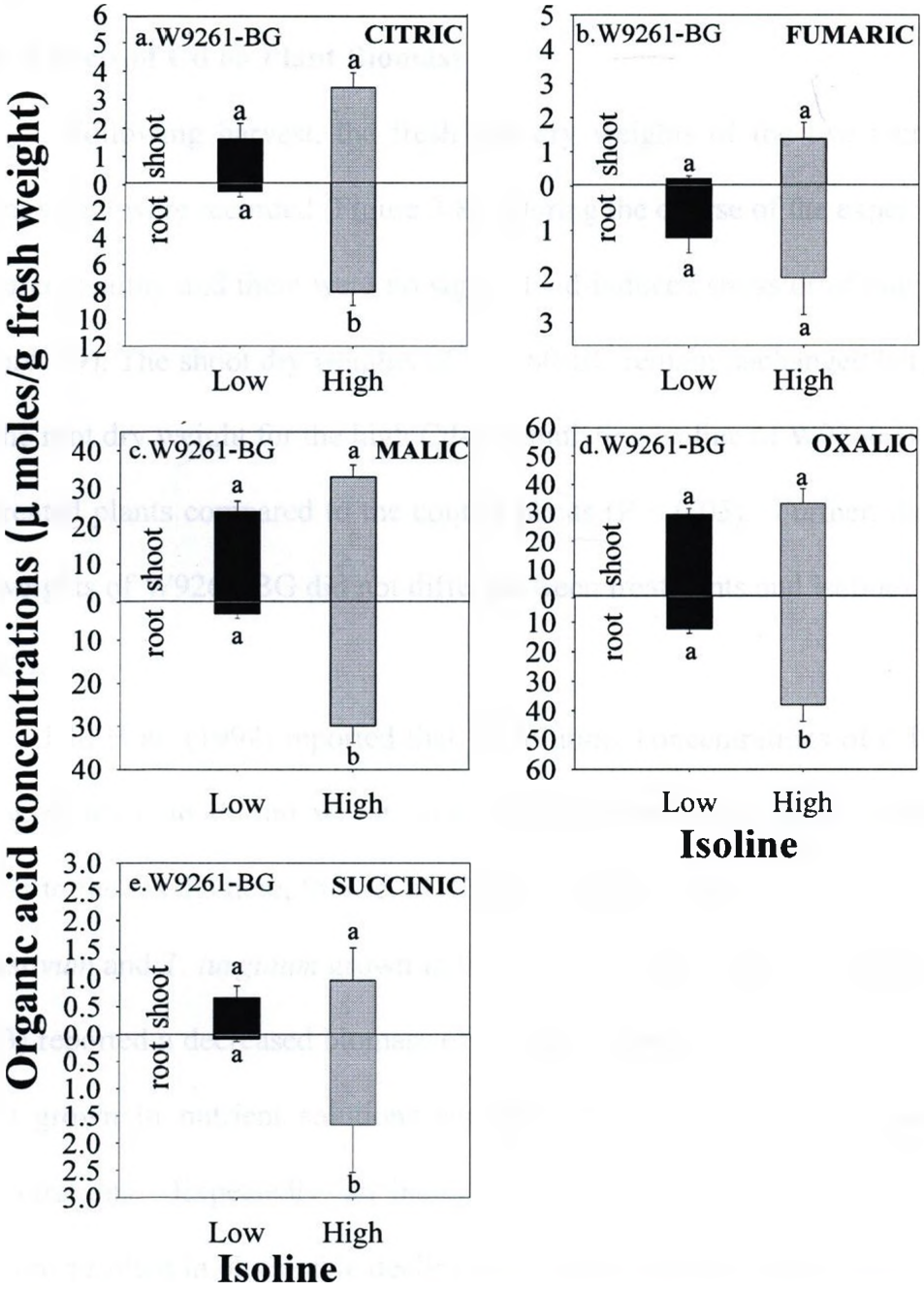


Figure 3.7: Concentrations of different organic acids in root and shoot tissues of W9261-BG grown in soil. Concentrations of (a) citric acid (b) fumaric acid (c) malic acid (d) oxalic acid and (e) succinic acid in the root and shoot tissues were determined by ion chromatography. Different lower case letters between isolines show significant difference ($P < 0.05$). Error bars represent SEM of four replicates.

3.2. Hydroponic Culture System

3.2.1. Effects of Cd on Plant Biomass

Following harvest, the fresh and dry weights of the two pairs of isolines of durum wheat were recorded (Figure 3.8). During the course of the experiments, all plants appeared healthy and there were no signs of Cd-induced stress or of nutrient deficiencies (Figure 3.9). The shoot dry weights of W9260-BC remain unchanged between treatments but the root dry weight for the high Cd-accumulating isoline of W9260-BC was greater in the treated plants compared to the control plants ($P < 0.05$). Further, the root and shoot dry weights of W9261-BG did not differ between treatments and isolines (Figure 3.8b) ($P > 0.05$).

Jalil et al. (1994) reported that, in solution, concentrations of Cd higher than 0.1 μM were toxic to durum wheat, and inevitably decreased plant biomass. However, similar to the results here, Stolt et al. (2003) found no effect of Cd on the dry weights of *T. aestivum* and *T. turgidum* grown in 0, 0.01 and 30 μM Cd. By contrast, Ozturk et al. (2003) reported a decreased biomass of two durum wheat cultivars (cvs Bacali-85 and C-1252) grown in nutrient solutions supplemented with 0, 6, 30, 75 and 150 μM Cd concentrations. Expectedly, an increased supply of Cd concentrations to the nutrient solutions resulted in observable decline in the shoot and root dry weights of both cultivars with C-1252 having a two-fold reduction in its shoot dry weight compared to the Bacali-85. Ozturk et al. (2003), like many others, used phytotoxic Cd concentrations which do not mimic what is realistic in the natural environments and this is evident with plants showing signs of acute Cd-induced stress.

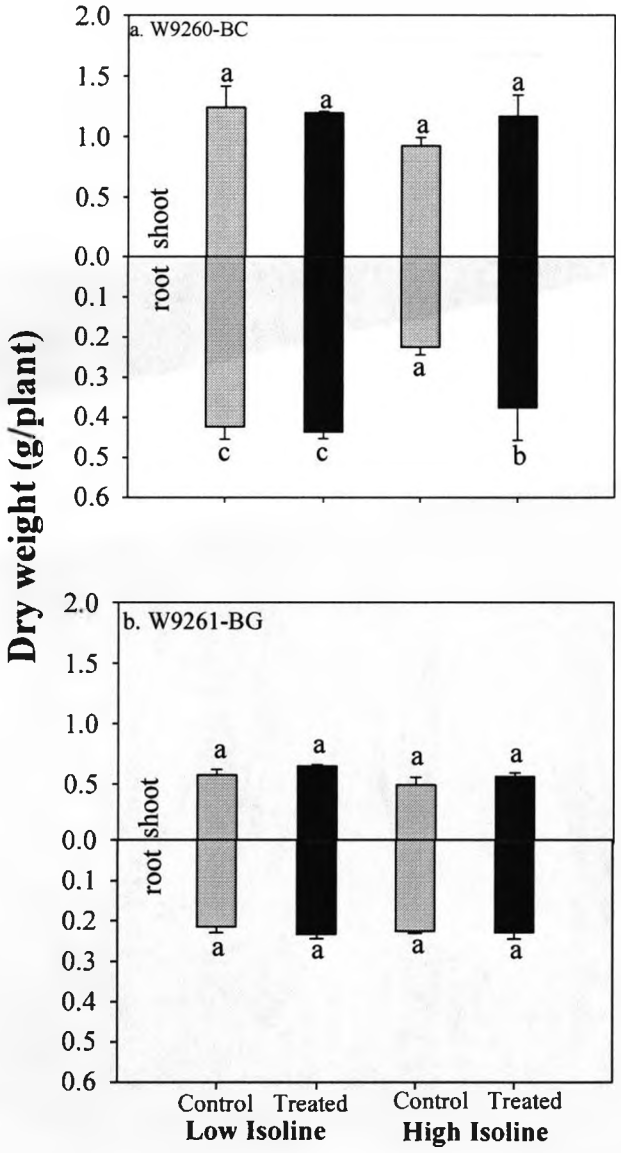


Figure 3.8: Effect of Cd on root and shoot dry weights of low and high Cd-accumulating isolines of durum wheat grown in hydroponics. Low and high Cd-accumulating isolines (a) W9260-BC and (b) W9261-BG were grown in hydroponic culture system for 16 d, harvested and oven-dried to constant weights. Different lower case letters denote significant differences ($P < 0.05$). Error bars represent SEM of three replicates.

Figure 3.9: Experimental set up for the hydroponically-grown plants. Plants appeared healthy throughout the experimental period and no signs of Cd-induced stress or nutrient deficiency were observed.

3.2.2. Cadmium Accumulation in Plant Tissues

Two pairs of isolines of durum wheat were investigated for their Cd content to validate the differential Cd accumulation that has been previously reported by several authors (Harris and Taylor, 2001, 2004; Hart et al., 2006). The Cd content in the shoot of the high Cd-accumulating isoline of W9260-BC was two-fold greater than that of low Cd-accumulating isoline when treated with 0.1 μM Cd (Figure 3.10a) ($P < 0.05$). No differences were found in the root of the low and high Cd-accumulating isolines of W9260-BC, indicating that root Cd influx is not a limiting factor and differential translocation of Cd from root to shoot must be responsible for differential accumulation of Cd in aboveground tissues. In W9261-BG, the Cd concentrations in the root and shoot tissues of the low and high Cd-accumulating isolines remain unchanged by treatment (Figure 3.10b). Lack of differential Cd accumulation in the W9261-BG isoline may be attributable to the low Cd content in their tissues. The trace amounts of Cd measured in the control plants of both pairs of isolines (Figure 3.10) are believed to be carry-over from Cd that was in the seed stock.

Differential Cd content in shoots of the low and high Cd-accumulating isolines have been previously reported (Harris and Taylor, 2001 and 2004; Stolt et al. 2006), and has been linked to restricted root-to-shoot Cd translocation in the low Cd-accumulating isoline. Findings of this study corroborate those of Harris and Taylor (2004) and Hart et al. (2006) who found no differences in the root Cd uptake between the pairs of isolines of 8982-TL and W9262-339A.

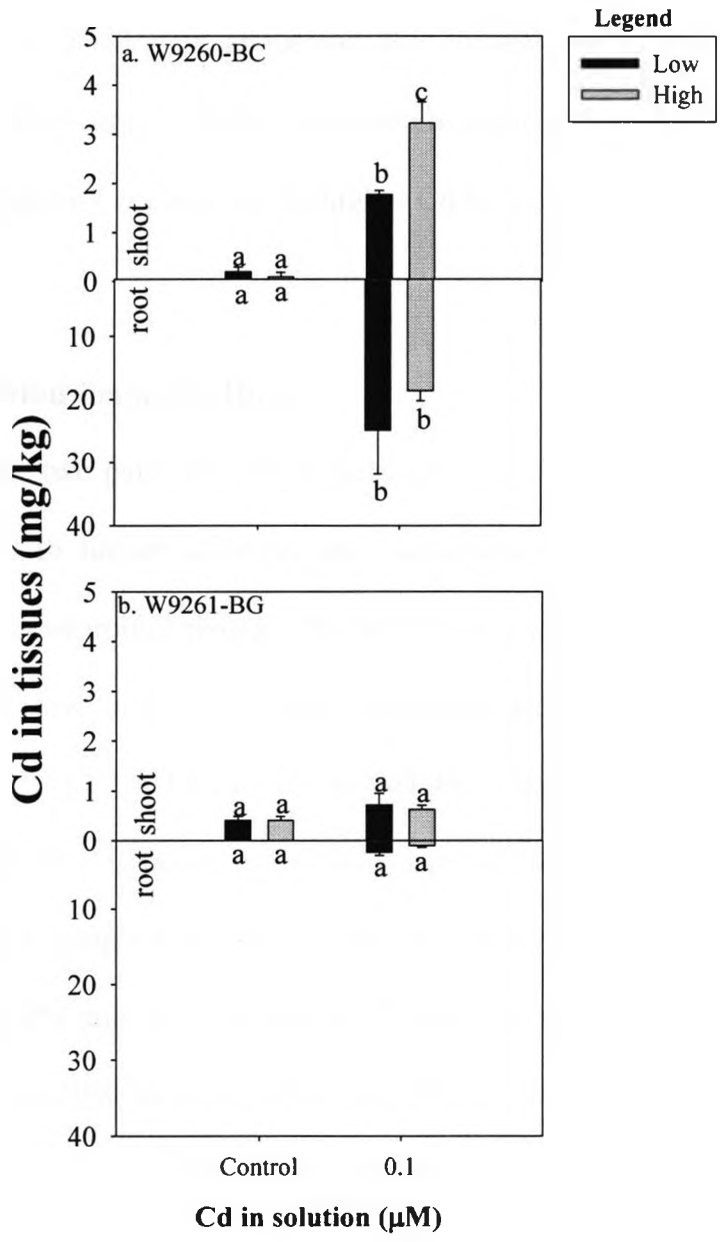


Figure 3.10: Concentrations of Cd in root and shoot tissues of low and high Cd-accumulating isolines of durum wheat grown in hydroponics. Low and high Cd-accumulating isolines (a) W9260-BC and (b) W9261-BG were grown in hydroponic culture system for 16 d, harvested, oven-dried to constant weights and analyzed for Cd using ICP-AES. Different lower case letters show significant differences between isolines ($P < 0.05$) and error bars denote SEM of three replicates.

The differential Cd accumulation in the shoots of W9260-BC here is in accord with Harris and Taylor (2001) who found that the Cd content in the grain of low Cd-accumulating isoline of 8982 was about two-fold lower than that of the high Cd-accumulating isoline; they suggested that Cd remobilization from the flag leaf of the high Cd-accumulating isoline may account for its higher Cd content.

3.2.3. Cadmium Distribution in the Root

The roots of the two pairs of isolines were investigated for Cd partitioning in the apoplast and symplast to further decipher the mechanisms behind the differential Cd accumulation in the aboveground tissues. In W9260-BC, the percentage Cd content in the root symplast of low and high Cd-accumulating isolines was 91% and 60%, respectively (Figure 3.11) ($P = 0.01$). In W9261-BG, the percentage Cd in the root symplast did not differ ($P = 0.88$) between isolines, with each having about 70% of the total root Cd in the root symplast (Figure 3.11b). In either case, the proportion of Cd in the root apoplast was 9% and 40% for the W9260-BC low and high Cd-accumulating isolines respectively, and 30% for both isolines of W9261-BG.

It has been reported that Cd in roots of *N. tabacum* and *T. caerulescens* was mostly associated with the cell wall (Boominathan et al., 2003), reflecting apoplastic binding, which is contrary to the findings of present study where Cd was mostly found in the root symplast. However, Perriguey et al. (2008) also found a large proportion of the

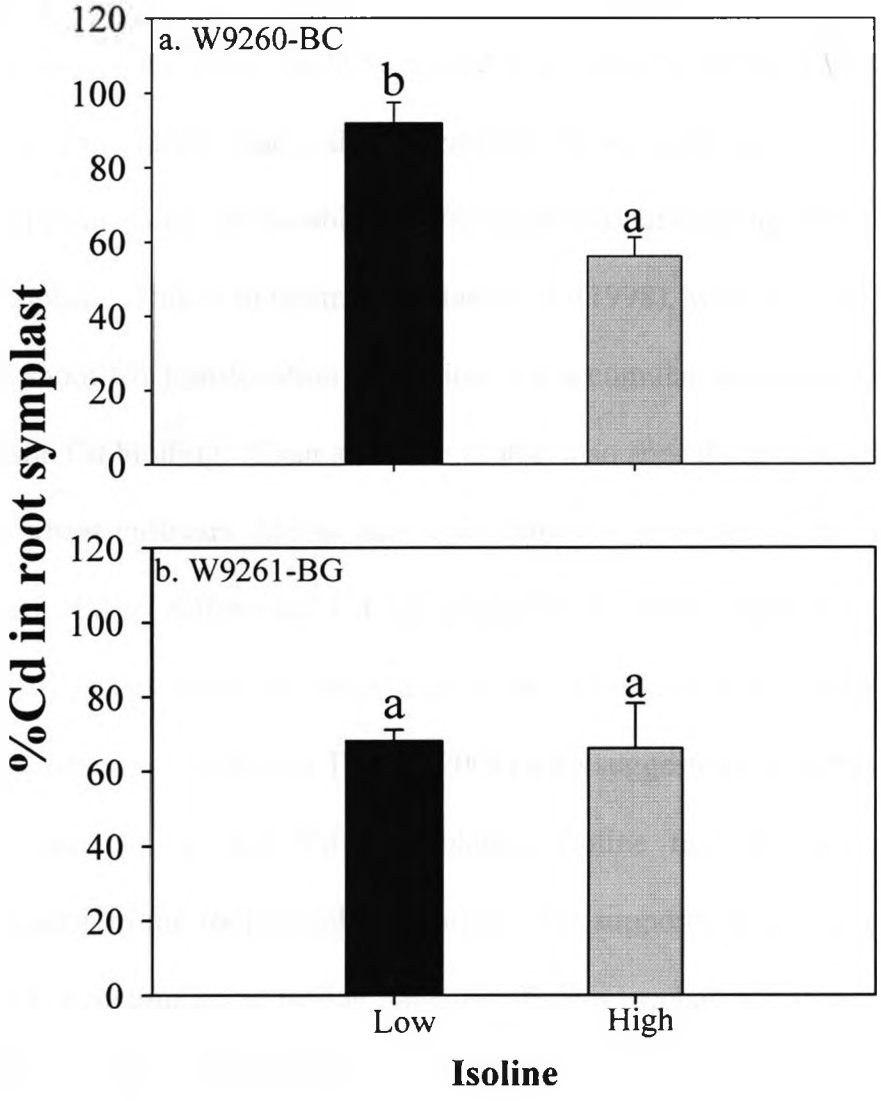


Figure 3.11: Percentage Cd in the root symplast of the low and high Cd-accumulating isolines of durum wheat. Low and high Cd-accumulating isolines (a) W9260-BC and (b) W9261-BG were grown in hydroponic culture system for 16 d, harvested and the roots washed with either distilled water (for total Cd in root) or 1 mM $CaSO_4$ to remove the extracellularly-bound Cd (apoplast). Dried root tissues were analyzed for Cd content using ICP-AES, and % Cd in the symplast was calculated using the equation [Cd in symplast/total Cd] x 100%. Different lower case letters denote significant difference between isolines ($P < 0.05$) and error bars denote SEM of three replicates.

Cd in maize root to be absorbed and stored in the symplast, hypothesizing that reduced translocation to the shoot might be related to a 'virtual selective gate' in the maize root.

In the current study, the differential Cd accumulation in the isolines of durum wheat appears to be attributable to differential Cd partitioning between the root apoplast and symplast. This is in contrast to Hart et al. (1998), who suggested that the decreased root to shoot Cd translocation in the low Cd-accumulating isoline may be linked to the apoplastic Cd binding. Chan and Hale (2004) also speculated that most of the root-Cd in durum wheat cultivars Arcola and Kyle plants is restricted to the apoplast, and linked apoplastic Cd to differential Cd accumulation between Arcola and Kyle. The current study provides evidence for root-Cd localization in the symplast, which is consistent with the hypothesis by Harris and Taylor (2004) who suggested that increased retention of Cd in the root of the low Cd-accumulating isoline may be associated with greater sequestration in the root symplast. Further, this supports the hypothesis that the roots of the low Cd-accumulating isoline have an efficient mechanism of retaining Cd in its root. The retention of Cd in the root of low Cd-accumulating isoline, especially in W9260-BC may be attributable to sequestration and/or complexation with organic ligands in the root symplast, possibly the vacuole.

3.2.4. Organic Acids in Exudates of Plants Grown Hydroponically

Exudation of organic acids into the nutrient solutions were monitored and quantified over a 6-day period (Figure 3.12). Oxalic acid was the predominant and only

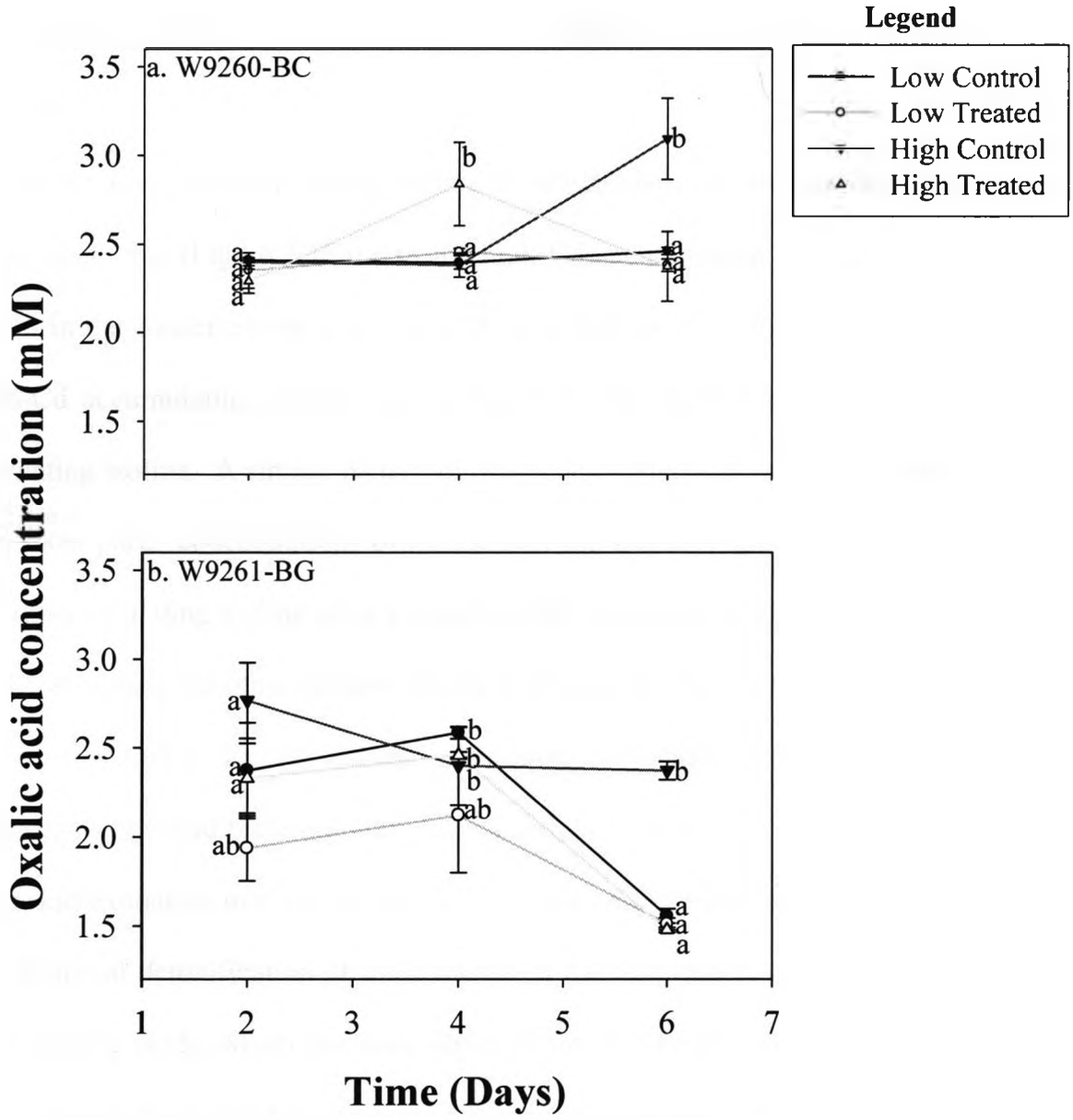


Figure 3.12: Time course of the exudation of oxalic acid in low and high Cd-accumulating isolines of durum wheat grown in hydroponics. The exudates from low and high Cd-accumulating isolines (a) W9260-BC and (b) W9261-BG were collected on days 2, 4 and 6 after Cd treatment and analyzed for organic acids using ion chromatography. Different lower case letters show significant differences at $P < 0.05$ and error bars denote SEM of four replicates.

detectable acid in the exudate of the isolines. The concentration of oxalic acid in the exudate ranged between 2.2 – 3.2 mM and 1.8 – 2.8 mM for W9260-BC and W9261-BG, respectively.

In the low Cd-accumulating isoline of W9260-BC, exudation of oxalic acid did not vary with time (Figure 3.12a). In the high Cd-accumulating isoline of W9260-BC, increases in the concentration of oxalic acid were seen on day 4 for the Cd-treated plants of high-Cd accumulating isoline, and on day 6 for the control plants of the high Cd-accumulating isoline. A similar pattern of oxalic acid exudation was seen in W9261-BG; for the most part, concentrations of oxalic acid did not vary greatly with time and the high Cd-accumulating isoline grown under control conditions had higher concentrations of oxalic acid than the other isolines on day 6 (Figure 3.12b). Similar results have been described previously for Al-resistant *Zea mays* (Kochian et al., 2005) where no differences were found between Al resistance and the exudation of citrate, suggesting that organic acid exudation may not be the only tolerance mechanism in maize.

External detoxification of toxic metals in the rhizosphere through chelation with exuded organic acids, which has been reported for Al-tolerant species (Miyasaka et al., 1991; Pellet et al., 1995; Ma et al., 1997), may not hold true for durum wheat as no significant differences were found in oxalic acid exudation between the low and high Cd-accumulating isolines treated with Cd. Thus, the differential Cd accumulation in these isolines may not be as a result of organic acids in the exudates but may be related to internal metal tolerance and/or sequestration.

3.2.5. Organic Acid Content in Durum Wheat Grown Hydroponically.

To better understand the differential Cd accumulation in isolines of durum wheat, organic acids in the tissues were analyzed and quantified with IC. The organic acids found in the two pairs of isolines examined were citric, fumaric, malic, oxalic and succinic acids.

In W9260-BC, total organic acids were higher in roots and shoots of the low Cd-accumulating isoline treated with Cd relative to their controls (Figure 3.13a). In contrast, total organic acids in the high Cd-accumulating isoline treated with Cd declined in the root and did not vary in the shoots compared to their respective controls. No differences in total organic acid concentration were found in roots and shoots of low and high Cd-accumulating isolines of W9261-BG treated with Cd relative to their controls (Figure 3.13b). A closer look into individual components of total organic acids is hereby presented for each pair of isoline.

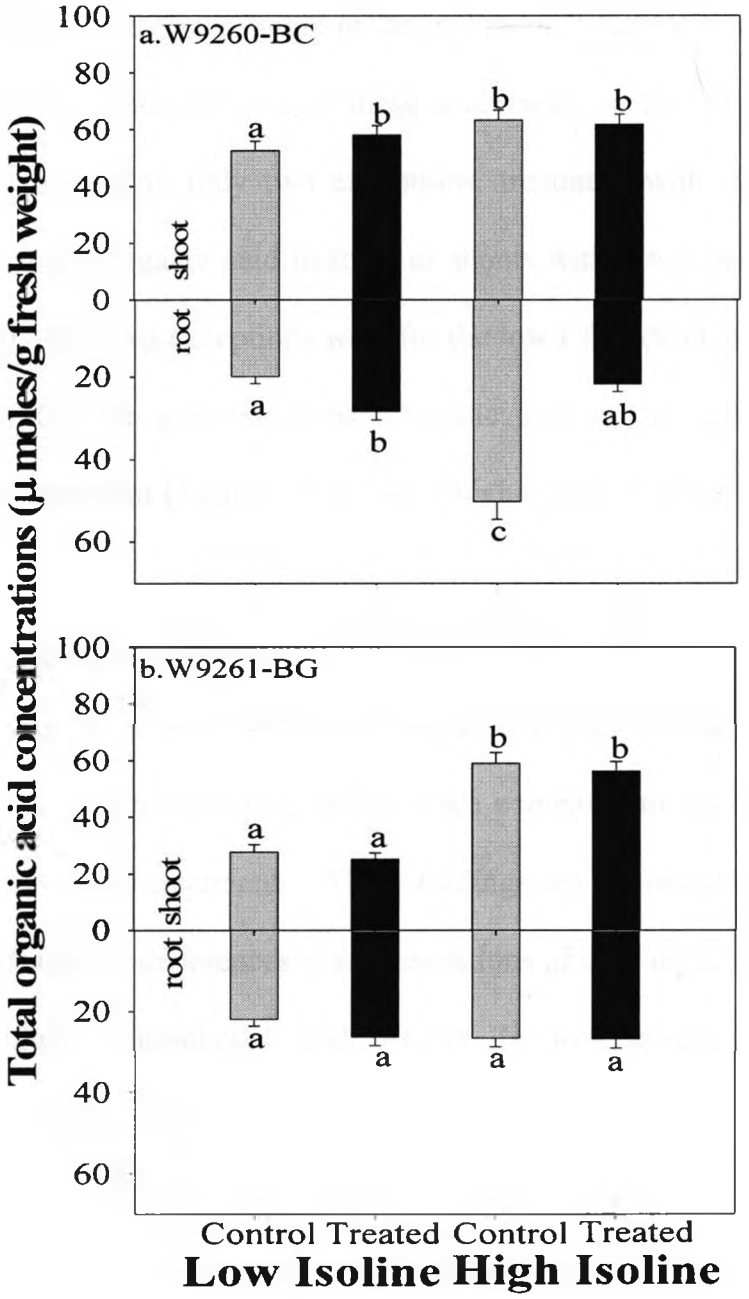


Figure 3.13: Total organic acid concentrations in root and shoot tissues of isolines of durum wheat grown in hydroponics. Low and high Cd-accumulating isolines (a) W9260-BC and (b) W9261-BG were grown in hydroponics for 16 d, harvested and analyzed for organic acid contents using ion chromatography. The total organic acids in root and shoot represent the sum of individual organic acid components found in the root and shoot of the isolines. Different lower case letters show significant differences within treatments ($P < 0.05$) and error bars denote SEM of four replicates.

3.2.5.1. W9261-BG Isolines

Four organic acids were found in the isoline of W9261-BG grown in hydroponic culture system, and concentrations of these acids were in the order oxalic > malic > fumaric = succinic. With only two exceptions, treatment with Cd did not affect the concentrations of any organic acid in roots or shoots within any pair of isolines (Figure 3.14) ($P > 0.05$). The two exceptions were for the low Cd-accumulating isoline grown in the presence of Cd; the concentrations of oxalic acid in the root (Figure 3.14c) and succinic acid in the shoot (Figure 3.14d) were higher than their respective controls ($P < 0.05$).

When comparing the low and high Cd-accumulating isolines, it is apparent that concentrations of fumaric acid, malic acid, oxalic acid and succinic acids were greater in shoots of the high Cd-accumulating isoline when compared to the low Cd-accumulating isoline, regardless of Cd-treatment. These findings are in contrast with Szmigielska et al. (2002) who found no differences in concentrations of organic acids in roots and shoots of Kyle (a high accumulator) and Arcola (a low accumulator) when grown hydroponically for two weeks.

In conclusion, the lack of differences in concentrations of organic acids in roots and shoots of W9261-BG grown in control solution as compared to those from plants treated with Cd may be related to the fact that the isolines did not differ in Cd content. This result is consistent with what was earlier reported for the soil-potted plants where no observable changes were found in Cd accumulation or organic acid content between the isolines (see Figure 3.1).

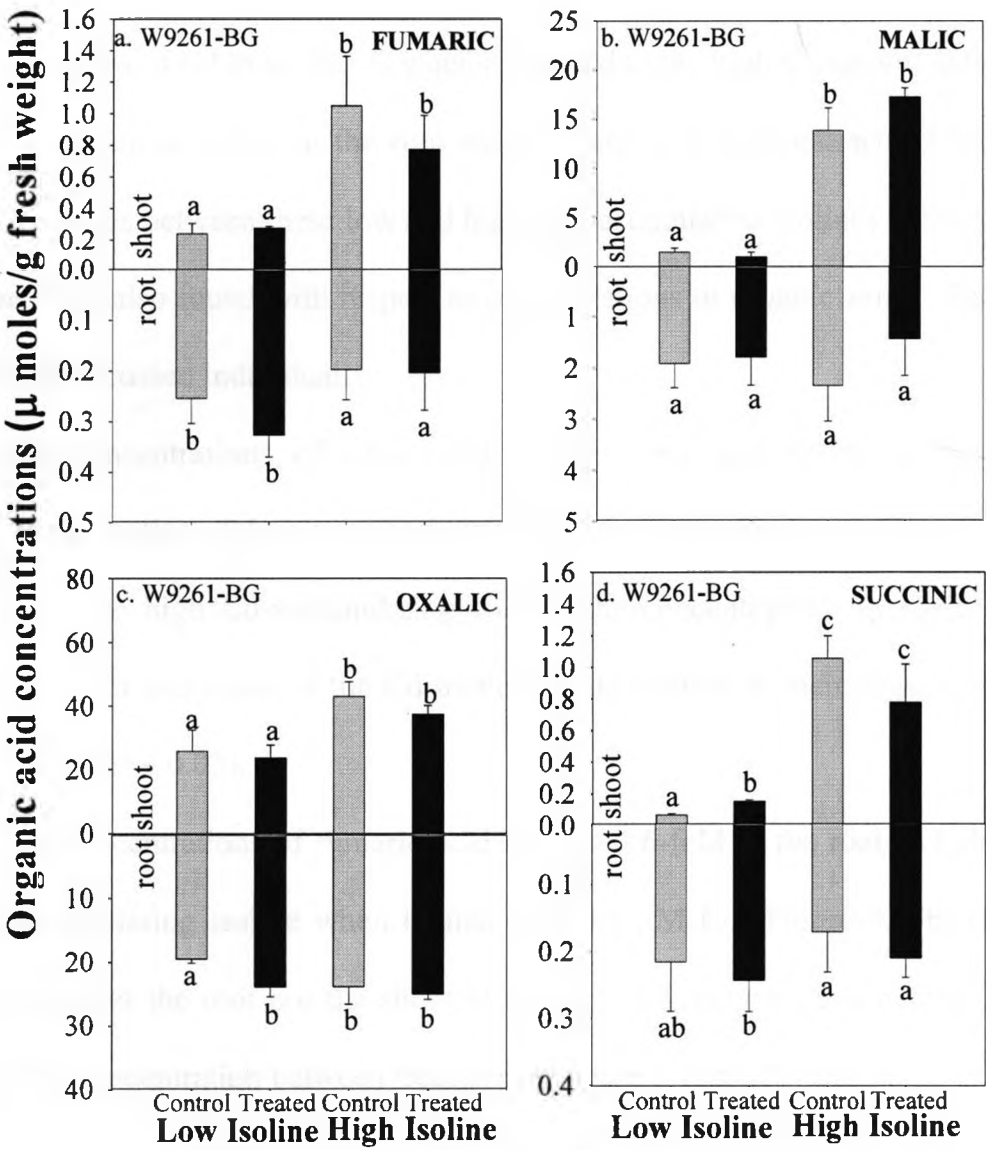


Figure 3.14: Concentrations of different organic acids in root and shoot tissues of W9261-BG grown in hydroponics. Concentrations of (a) fumaric acid (b) malic acid (c) oxalic acid and (d) succinic acid in the root and shoot tissues of W9261-BG grown in hydroponic culture system for 16 d were quantified using ion chromatography. Different lower case letters between treatments show significant difference ($P < 0.05$). Error bars represent SEM of four replicates.

3.2.5.2. W9260-BC Isolines

In this pair (W9260-BC), the low Cd-accumulating isoline had approximately half the concentration of Cd in its leaves when compared to the high Cd-accumulating isoline, while concentrations of Cd in the root did not vary with Cd treatment (Figure 3.10a). Some differences between these low and high Cd-accumulating isolines in response to Cd treatment were also found with respect to concentrations of organic acids. Each organic acid will be discussed individually.

The concentrations of citric acid in the roots and shoots of the low Cd-accumulating isoline did not vary between treatments (Figure 3.15a) ($P > 0.05$). However, in the high Cd-accumulating isoline, the concentrations of citric acid were lower in the root and shoot of the Cd-treated plants relative to their respective controls. (Figure 3.15a) ($P < 0.05$).

The concentrations of fumaric acid increased 6-fold in the root and shoot of the low Cd-accumulating isoline when treated with 0.1 μM Cd (Figure 3.15b) ($P < 0.05$). However, neither the root nor the shoot of the high Cd-accumulating isoline differed in fumaric acid concentration between treatments (Figure 3.15b) ($P > 0.05$).

In the low Cd-accumulating isoline, the concentrations of malic acid were greater in the root of Cd-treated plant (Figure 3.15c) ($P < 0.05$) while malic acid in shoots remain unchanged between treatments (Figure 3.15c) ($P > 0.05$). However, the concentrations of malic acid in roots and shoots of high Cd-accumulating isoline did not vary between control and treatment (Figure 3.15c) ($P > 0.05$).

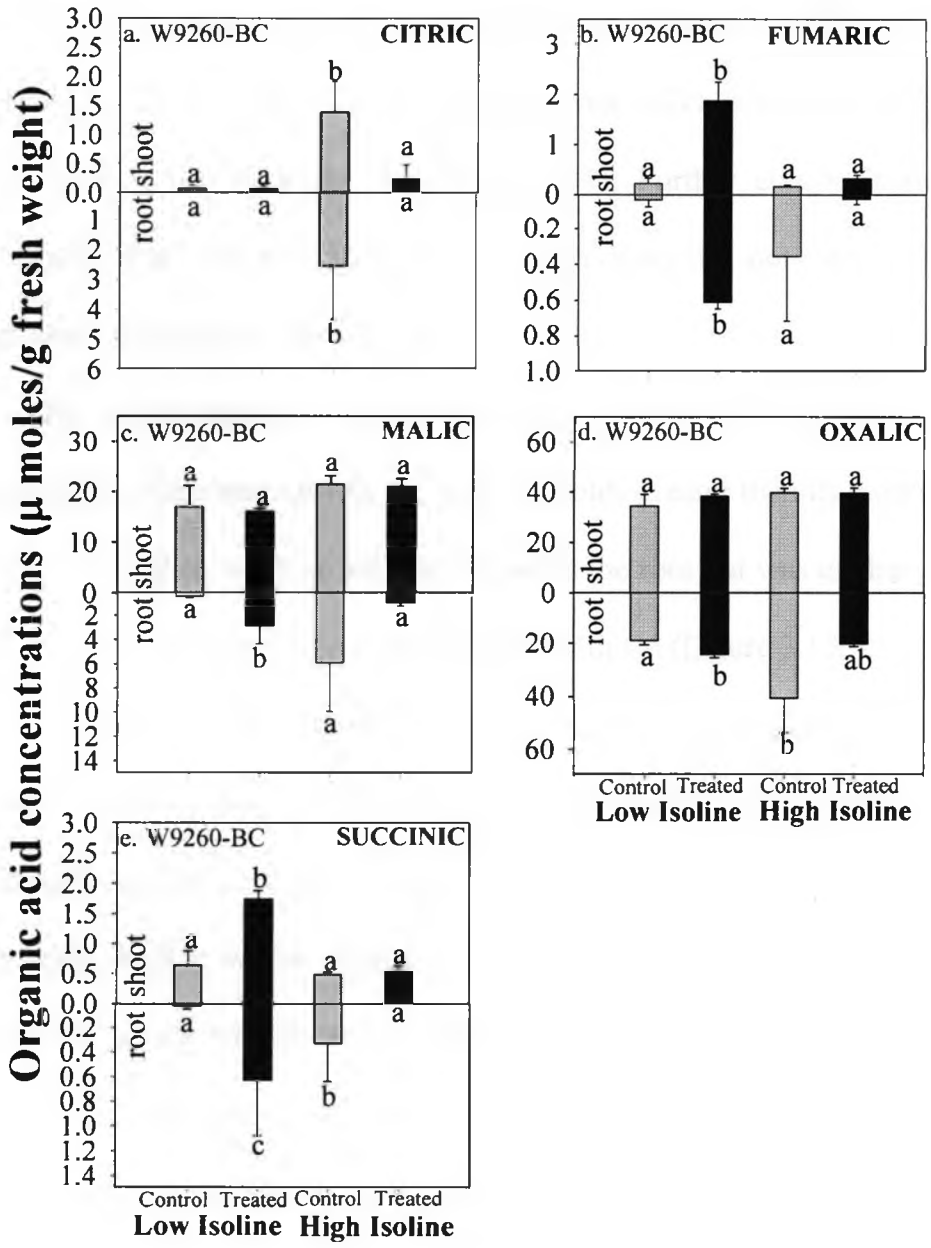


Figure 3.15: Concentrations of different organic acids in root and shoot tissues of W9260-BC grown in hydroponics. Concentrations of (a) citric acid (b) fumaric acid (c) malic acid (d) oxalic acid and (e) succinic acid in the root and shoot tissues of W9260-BG grown in hydroponic culture system for 16 d were quantified using ion chromatography. Different lower case letters between treatments show significant difference ($P < 0.05$). Error bars represent SEM of four replicates.

The concentration of oxalic acid in the root of low Cd-accumulating isoline was greater in the Cd-treated tissue relative to control. (Figure 3.15d) ($P < 0.05$). However, the concentration of oxalic acid in shoots did not differ between treatments of the low Cd-accumulating isoline (Figure 3.15d) ($P > 0.05$). Further, concentrations of oxalic acid in roots and shoots of high Cd-accumulating isoline did not vary from control when treated with Cd (Figure 3.15d) ($P > 0.05$).

The concentrations of succinic acid in the root and shoot of the low Cd-accumulating isoline treated with Cd were 3-6 folds greater than its control (Figure 3.15e) ($P < 0.05$). Further, succinic acid decreased in the root but was unchanged in the shoots of high Cd-accumulating isoline when treated with Cd (Figure 3.15e).

In summary, concentrations of fumaric, malic, oxalic and succinic acids in the root, and of fumaric and succinic acids in the shoot of the low Cd-accumulating isoline were greater in the Cd-treated tissue. Contrasting results were found in high Cd-accumulating isoline where concentrations of citric acid in the root and shoot, and of succinic acid in the root decreased in the Cd-treated tissue. Overall, as seen in Figure 3.13a, significant increase in the total organic acid concentrations in the root and shoot of the low Cd-accumulating isoline treated with Cd is a reflection of increases in components of individual organic acids in Figure 3.15. Further, a decline in the total organic acid concentration in the root of high Cd-accumulating isoline suggests that decreases reported for citric acid (Figure 3.15a) and succinic acid (Figure 3.15e) are important in terms of the total pool of organic acids.

3.2.5.3. Organic acids as chelators?

In the present study, greater concentrations of organic acids in the root and shoot of low Cd-accumulating isoline treated with Cd compared to the control plants (Figure 3.13) suggest that organic acids may be employed as mechanism of metal chelation in the low Cd-accumulating isoline. Also, it is interesting that the low Cd-accumulating isoline with more Cd in its root symplast (Figure 3.11a) produced more organic acids overall in its root (Figure 3.13a), indicating that Cd could be complexed with organic acids, and possibly sequestered in the vacuole. Thus, the Cd-organic acid complexes sequestered in the root vacuole might be attributable to the restricted Cd translocation from root to shoot in the low Cd-accumulating isoline. Also, though not tested in this study, there is a possibility that the increased organic acids measured in the shoot of the low Cd-accumulating isoline might form complexes with Cd and thus restrict Cd loading into the grains. Similar results have been reported by Guo et al. (2007) who found an increased production in organic acids in maize (*Zea mays* L.) roots treated with Cd, suggesting that organic acids produced might be involved in Cd tolerance and detoxification.

Similar to the studies on isolines of durum wheat, organic acids have been implicated in Zn hyperaccumulation and tolerance of *Arabidopsis halleri* (Zhao et al., 2000). They found no significant differences in concentrations of citric and malic acids in shoots but a positive increase in concentrations of both acids in the root of *A. halleri* treated with Zn. In contrast, Nakamura et al. (2008) detected citric and malic acids in the xylem exudate of oilseed rape (*Brasicca napus* L.), providing inconclusive evidence for citric acid and malic acid as Cd chelators in the xylem exudate. In durum wheat, reduced root-to-shoot Cd translocation in low Cd-accumulating isoline has been associated with a

lower Cd concentration in the xylem sap and reduced xylem sap exudation (Harris and Taylor, 2004) implying that Cd in the root cells might have been sequestered and complexed with organic acids (Figure 3.13a).

3.3. Summary and Conclusions

In this present study, attempts were made to decipher physiological and biochemical mechanisms governing the differential Cd accumulation in two of the five pairs of isolines of durum wheat derived by Clarke et al. (1997). This differential Cd accumulation has been associated with several factors including greater retention of Cd in the roots of the low Cd-accumulating isoline (Chan and Hale, 2004; Harris and Taylor, 2004; Hart et al., 2005, 2006), higher translocation of Cd from root to shoot in the high Cd-accumulating isoline (Harris and Taylor, 2004; Stolt et al., 2003), and greater root surface area in the low Cd-accumulating cultivar, Arcola (Berkelaar and Hale, 2000). However, none of these studies provided conclusive evidence for the mechanism governing differential Cd accumulation in the isolines of durum wheat but have suggested that this differential accumulation may be attributable to root functioning rather than differences in root-Cd influx.

Other factors that may be responsible for differential Cd accumulation in the isolines of durum wheat are phytochelatins and organic acids. Previous studies (Hart et al., 2006; Stolt et al., 2003 and Bahrami, 2006) revealed no clear correlation between concentrations of Cd and phytochelatin. Thus, the present study focused mainly on the possible roles of organic acids in the plant to decipher the mechanisms of differential Cd

accumulation. Soil and hydroponic culture systems were adopted in this study. In the soil culture systems, Cd accumulations did not differ between the low and high Cd-accumulating isolines and this may be attributable to low extractable Cd in the soil.

Further, there were no differences between low and high Cd-accumulating isolines in the total organic acids produced except in the high Cd-accumulating isoline of W9261-BG, which had greater organic acids in its root than did the low Cd-accumulating isoline. However, organic acids measured in soil culture experiments may not be associated with Cd uptake and translocation as no differences in Cd accumulation were detected. The experiments were taken further in the hydroponic culture systems to determine the relative contributions of internal (symplastic) versus external (apoplastic) immobilization of Cd in roots. Cd was mostly found in the root symplast, with the low Cd-accumulating isoline of W9260-BG having a greater percentage Cd than high-Cd accumulating isoline, suggesting that Cd is mostly bound to the symplast and not to the apoplast as suggested by Hart et al. (1998).

External detoxification of Cd by organic acids does not seem to be an important factor in the present study as no differences were found in organic acids exuded by the two pairs of isolines grown in soil or hydroponic culture systems. However, total organic acids in tissue revealed different patterns for the two pairs of isolines. The isolines of W9261-BG, with no differential Cd accumulation in either root or shoot, showed no differences in the total organic acids in its root and shoot. However, in W9260-BC, total organic acids were greater in the root and shoot of the low Cd-accumulating isoline treated with Cd while there was a decrease in the total organic acids in the root of the high Cd-accumulating isoline, suggesting that higher Cd in the root symplast of the low

Cd-accumulating isoline (Figure 3.11a) may be associated with greater concentration of total organic acids in the root (Figure 3.13a). It is possible that the Cd-organic acid complexes are sequestered in the root vacuole (see Figure 1.2), thus resulting in decreased Cd translocation from root to shoot in the low Cd-accumulating isoline.

Further studies will be required to elucidate the cellular localization of Cd in the root symplast of the low Cd-accumulating isoline. Also, enzymes (see Figure 1.3) involved in organic acid production could be investigated to assess their possibilities as bioindicators of the differential Cd response.

Finally, four organic acids at different concentrations were found in W9261-BG and five in W9260-BC supporting the hypothesis that low and high Cd-accumulating isolines from near isogenic pairs of *T. turgidum* var *durum* will produce different types and concentrations of LMWOAs when grown in the presence of Cd.

Based on the experimental observations from this present study, it can be concluded that reduced translocation of Cd to the leaves of the low Cd-accumulating isoline, especially in W9260-BC, was associated with greater retention of Cd in the root symplast, likely as a result of chelation with citric, malic, oxalic and/or succinic acids.

REFERENCES

AAFC (Agriculture and Agri-Food Canada). 2007. Wheat: situation and outlook. Bi-weekly Bulletin. 20:1-6.

Archambault, D.J., Marentes, E., Buckley, W., Clarke, J., and Taylor, G.J. 2001. A rapid, seedling-based bioassay for identifying low cadmium-accumulating individuals of durum wheat (*Triticum turgidum* L.). Euphytica. 117: 175-182.

Bahrami, S. 2006. Differential accumulation of cadmium in durum wheat: roles of phytochelatins. MSc thesis. Dept. of Biology. University of Western Ontario, London, Ontario.

Balestrasse, K.B., Gardey, L., Gallego, S.M., and Tomaro, M.L. 2001. Response of antioxidant defence system in soybean nodules and roots subjected to cadmium stress. Australian Journal of Plant Physiology. 28: 497-504.

Benavides, M.P., Gallego, S.M., and Tomaro M.L. 2005. Cadmium toxicity in plants Brazilian Journal of Plant Physiology. 17: 21-34.

Berkelaar, E., and Hale, B. 2000. The relationship between root morphology and cadmium accumulation in seedlings of two durum wheat cultivars. Canadian Journal of Botany. 78: 381-387.

Bjorck, J., Granfeldt, Y., Liljeberg, H., Tovar, J., and Asp, N.G. 1994. Food properties affecting the digestion and absorption of carbohydrates. American Journal of Clinical Nutrition. 59: 669-705.

Boominathan, R., and Doran, P.M. 2003. Organic acid complexation, heavy metal distribution and the effect of ATPase inhibition in hairy roots of hyperaccumulator plant species. Journal of Biotechnology. 101: 131-146.

CCME (Canadian Council of Ministers of the Environment). Canadian soil quality guidelines for the protection of environmental human health [online]. Available from http://www.ccme.ca/assets/pdf/pn_1268_e.pdf [updated 2006; cited June 2008]

Chan, D.Y. and Hale, B.A. 2004. Differential accumulation of Cd in durum wheat cultivars: uptake and retranslocation as sources of variation. Journal of Experimental Botany. 55: 2571-2579.

Chiang, P.N., Wang, M.K., Chiu, C.Y., and Chou, S.Y. 2006. Effects of cadmium amendments on low-molecular-weight organic acid exudates in rhizosphere soils of tobacco and sunflower. Environmental Toxicology. 21: 479 – 488.

Cho, U.H., Sohn, J.Y. 2004. Cadmium-induced changes in antioxidative systems, hydrogen peroxide content, and lipid peroxidation in *Arabidopsis thaliana.* Journal of Plant Biology. 47: 262-269.

Cieśliński, G., Van Rees, K.C.J., Szmigielska, A.M., and Huang, P.M. 1997. Low molecular-weight organic acids released from roots of durum wheat and flax into sterile nutrient solutions. Journal of Plant Nutrition. 20: 753-764.

Cieśliński, G., Van Rees, K.C.J., Szmigielska, A.M., Krishnamurti, G.S.R., and Huang, P.M. 1998. Low-molecular-weight organic acids in rhizosphere soils of durum wheat and their effect on cadmium bioaccumulation. Plant and Soil 203: 109–117.

Clarke, J.M., Leisle, D., and Kopytko, G.L. 1997. Inheritance of cadmium concentration in five durum wheat crosses. Crop Science. 37: 1722-1726.

Clarkson, D.T. 1993. Roots and the delivery of solutes to the xylem. Philosophical Transactions of the Royal Society, London, Series B. 341: 5-17.

Clemens, S. 2001. Molecular mechanisms of plant metal tolerance and homeostasis. Planta. 212: 475-486.

Cobbett C.S. 2001. Heavy metal detoxification in higher plants: phytochelatin synthesis and function. International Union of Biochemistry and and Molecular Biology Life. 51: 183-188.

Cobbett, C.S. 2000. Phytochelatins and their roles in heavy metal detoxification. Plant Physiology. 123: 825-832.

Codex Alimentarius Commission. 2005. Joint FAO/WHO food Standards Programme. Twenty-Eight Session, Rome, Italy 4-9 July 2005. Report of the 37th session of the Codex Committee on food Additives and Contaminants, 25-29 April 2005. Para. 175, Appendix XXVI. The Hague, the Netherlands. http://www.codexalimentarius.net/web/reports.jsp]ALINORM05/28/12. Accessed March 16, 2007.

Das, P., Samantaray, S., and Rout, G.R. 1997. Studies on cadmium toxicity in plants: a review. Environmental Pollution. 98: 29-36.

Delhaize, E., Ryan, P.R., and Randall, P.J. 1993. Aluminium tolerance in wheat (*Triticum aestivum* L.). Plant Physiology. 103: 695-702.

Egle, K., Römer, W., and Keller, H. 2003. Exudation of low molecular weight organic acids by *Lupinus albus* L., *Lupinus angustifolius* L. and *Lupinus luteus* L. as affected by phosphorus supply. Agronomie. 23: 511-518.

Frank, R., Ishida K., and Suda, P. 1976. Metals in agricultural soils of Ontario. Canadian Journal of Soil Science. 56: 181-196.

Garrett, R.G., MacLaurin, A.L., Gawalko, R., Tkachuk, R., and Hall, G.E.M. 1998. A prediction model for estimating the cadmium content of durum wheat for soil chemistry. Journal of Geochemical Exploration. 64: 101-110.

Ghosh, M., and Singh, S.P. 2005. A comparative study of cadmium phytoextraction by accumulator and weed species. Environmental Pollution. 133: 365-371.

Grant, C.A., and Sheppard, S.C. 2008. Fertilizer impacts on cadmium availability in agricultural soils and crops. Human and Ecological Risk Assessment. 14: 210-228.

Guo, B., Peng, Z.., Han, F., Shan, X., and Lin, J. Study of low molecular weight organic acids in maize roots under the stress of cadmium using capillary zone electrophoresis. Journal of Separation Science. 2742-2747.

Han, F., Shan, X-Q., Zhang, J., Xie, Y-N, Pei, Z-G., Zhang, X-Z., Zhu, Y-G., and Wei, B. 2005. Organic acids promote the uptake of lanthanum by barley root. New Phytologist. 165: 481-492.

Harris, N.S. and Taylor, G.J. 2001. Remobilization of cadmium in maturing shoots of near isogenic lines of durum wheat that differ in grain cadmium accumulation. Journal of Experimental Botany. 52: 1473-1481.

Harris, N.S. and Taylor, G.J. 2004. Cadmium uptake and translocation in seedlings of near isogenic lines of durum wheat that differ in grain cadmium accumulation. BioMed Central Plant Biology. 4: 4-15.

Hart, J.J., Welch, R.M., Norvel, W.A., Clarke, J.M., and Kochian, L.V. 2005. Zinc effects on cadmium accumulation and partitioning in near-isogenic lines of durum wheat that differ in grain cadmium concentration. New Phytologist. 167: 391-401.

Hart, J.J., Welch, R.M., Norvell, W.A., and Kochian, L.V. 2006. Characterization of cadmium uptake, translocation and storage in near- isogenic lines of durum wheat that differ in grain cadmium accumulation. New Phytologist. 172: 261-271.

Hart, J.J., Welch, R.M., Norvell, W.A., Sullivan, L.A., and Kochian, L.V. 1998. Characterization of cadmium binding, uptake, and translocation in intact seedlings of bread and durum wheat cultivars. Plant Physiology. 116: 1413-1420.

Heiny, J.S., and Tate C.M., 1997. Concentrations, distribution and comparison of selected trace elements in bed sediment and fish tissue in the South Platte River basin, USA, 1992-1993. Environmental Contamination and Toxicology. 32: 246-259.

Heiss, S., Wachter A., Bogs J., Cobett C., and Rausch T. 2003. Phytochelatin synthase (PCS) protein is induced in *Brassica juncea* leaves after prolonged cadmium exposure. Journal of Experimental Botany. 54: 1883-1839.

Hernandez, L.E., Carpena-Ruiz, R., and Grant, A. 1996. Alterations in the mineral nutrition of pea seedlings to cadmium. Journal of Plant Nutrition. 19: 1581-1598.

Jalil, A., Selles, F., and Clarke, J.M. 1994. Effect of cadmium on growth and uptake of cadmium and other elements by durum wheat. Journal of Plant Nutrition. 17: 1839-1858.

Johnson, J.F., Allan, D.L., and Vance, C.P. 1994. Phosphorus stress-induced proteoid roots show altered metabolism in *Lupinus albus*. Plant Physiology. 104: 657-665.

Jones, D.L., Darrah, P.R., and Kochian, L.V. (1996) Critical-evaluation of organic acid mediated iron dissolution in the rhizosphere and its potential role in root iron uptake. Plant and Soil. 180: 57-66.

Jones, D.L. 1998. Organic acids in the rhizosphere- a critical review. Plant and Soil 205: 25-41.

Kochian, L.V. 1995. Cellular mechanisms of aluminium toxicity and tolerance in plants. Annual Review of Plant Physiology and Plant Molecular Biology. 46: 237-260.

Liu, D., Jiang, W., Wang, W., and Zhai, L. 1995. Evaluation of metal ion toxicity on root tip cells by the *Allium* test. Israel Journal of Plant Science. 43: 125-133.

Liu, J., Qian, M., and Cai, G. 2007. Variations between rice cultivars in root secretion of organic acids and the relationship with plant cadmium uptake. Environmental Geochemistry and Health. 29: 189-195.

López-Bucio, J., Nieto-Jacobo, M.F., Ramirez-Rodriguez V., and Herrera-Estrella L. 2000. Organic acids metabolism in plants: from adaptive physiology to transgenic varieties for cultivation in extreme soils. Plant Science. 160: 1-13

López-Bucio, J., Cruz-Ramírez, A., and Herrera-Estrella, L. 2003. The role of nutrient availability in regulating root architecture. Current Opinion in Plant Biology. 6: 280-287.

Lynch, J.P. 2007. Roots of the second revolution. Australian Journal of Botany. 55: 493-512.

Ma, J.F., Zheng, S.J., and Matsumoto, H. 1997. Specific secretion of citric acid induced by Al stress in *Cassia tora* L. Plant Cell Physiology. 28: 1019-1025.

McCutcheon, M.E. 2003. Response of near-isogenic lines of *Triticum turgidum* var *durum* to cadmium stress: low molecular weight organic acids as a tolerance mechanism. Undergraduate thesis. University of Western Ontario, London, Ontario.

McLaughlin, M.J., Parker D.R., and Clarke, J.M., 1999. Metals and micronutrients- food safety issues. Field Crop Research. 60: 143-163.

Mirshra, S., Sirvastave, S., Tripathi, R.D., Govindarajan, R., Kuriakose, S.V., and Prasad, M.N.V. 2006. Phytochelatin synthesis and response of antioxidants during cadmium stress in *Bacopa monnieri* L. Plant Physiology and Biochemistry. 44: 25-37.

Miyasaka, S.C., Buta, J.G., Howell, R.K., and Foy, C.D. 1991. Mechanisms of aluminium tolerance in snapbeans. Plant Physiology. 96: 737-743.

Nakamura, S., Akiyama, C., Sasaki, T., Hattori, H., and Chino, M. 2008. Effect of cadmium on the chemical composition of xylem exudate from oilseed rape plants (*Brassica napus* L.). Soil Science and Plant Nutrition. 54: 118-127.

Nigam, R., Srivastava, S., Prakash, S., and Srivastava, M.M. 2000. Effect of organic acids on the availability of of cadmium in wheat. Chemical Speciation and Bioavailabilty. 23: 125-132.

Oberlunder, H.E., and Roth K. 1978. As cited in M. Ross (ed.) Toxic Metals in Soil-Plant System (1994) John Wiley and Sons, New York.

Ortiz, D.F., Ruscitti, T., McCue, K.F., and Ow, D.W. 1995. Transports of metal binding peptides by HMT1, a fission yeast ABC-type vacuolar membrane protein. Journal of Biological Chemistry. 270: 4721-4728.

Ozturk, L., Eker, S, Ozkutlu, F., and Cakmak, I. 2003. Effct of cadmium on growth and concentrations of cadmium, ascorbic acid and sulphydryl group in durum wheat cultivar. Turkey Journal of Agriculture. 27: 161-168.

Pellet, D.M., Grunes, D.L., and Kochian, L.V. 1995. Organic acid exudation as an aluminium-tolerance mechanism in maize (Zea mays L.). Planta. 196: 788-795.

Perriguey, J., Sterckema, T., and Morel, J. 2008. Effect of rhizosphere and plant-related factors on the cadmium uptake by maize (*Zea mays* L.). Environmental and Experimental Botany. 63: 333-341.

Piñeros, M.A., Shaff, J.E., Manslank, H.S., Alves, V.M.C., and Kochian, L.V. 2005. Aluminium resistance in maize cannot be solely explained by root organic acid exudation. A comparative physiological study. Plant Physiology. 137: 231-241.

Prasad, M.N.V. 1995. Cadmium toxicity and tolerance in vascular plants. Environmental and Experimental Botany. 35: 525-545.

Prasad, M.N.V. and Freitas, H.M. 2003. Metal hyperaccumulation in plants- biodiversity prospecting for phytoremediation technology. Electronic Journal of Plant Biotechnology. 6: 285-321.

Russell, P.R., Wolfe, S.L., Hetz, P.E., Starr, C., and McMillan, B. 2008. Biology: The dynamic science. Volume 1. (1st Ed.) Thomson Brooks. Belmont, California, USA.

Salisbury, F.B., and Ross, C.W. 1985. Plant Physiology. (3rd Ed.) Wadsworth Inc. California, USA.

Sandalio L.M., Dalurzo H.C., Gomez M., Romero- Puertas M.C., and del Rio L.A. 2001. cadmium-induced changes in the growth and oxidative metabolism of pea plants. Journal of Experimental Botany. 52: 2115- 2126.

Sanita di Toppi, L., and Gabrielli R., 1999. Response to cadmium in higher plants. Environmental and Experimental Botany. 41: 105-130.

Sanita di Toppi, L., Vurro, E., Rossi, L., Marabottini, R., Musetti, R., Careri, M., Maffini, M., Mucchino, C., Corradini, C., and Badiani, M. 2007. Different compensatory mechanisms in two metal-accumulating aquatic macrophytes exposed to acute cadmium stress in outdoor artificial lakes. Chemosphere. 68: 769-780.

Sarret, G., Saumitou-Laprade, P., Bert, V., Proux, O., Hazemann, J-L., Traverse, A., Marcus, M.A., and Manceau, A. 2002. Forms of zinc accumulated in the hyperaccumulator *Arabidopsis halleri*. Plant Physiology. 130: 1815-1826.

Shah, K., Dubey, R.S. 1995. Effect of cadmium on RNA level as well as activity and molecular forms of ribonuclease in growing rice seedlings. Plant Physiology and Biochemistry. 33: 577-584.

Stolt, J.P., Asp, H., and Hultin, S. 2006. Genetic variation in wheat cadmium accumulation on soil with different cadmium concentrations. Journal of Agronomy and Crop Science. 192: 201-208.

Stolt, J.P., Sneller, F.E.C., Bryngelsson, T., Lundborg, T., and Schat, H. 2003. Phtochelatin and cadmium accumulation in wheat. Environmental and Experimental Botany. 49: 21-28.

Sun, R., Zhou, Q., and Jin, C. 2006. Cadmium accumulation in relation to organic acids in leaves of *Solanum nigrum* L. as a newly found cadmium hyperaccumulator. Plant and Soil. 285: 125-134.

Sweeney, M.N. 2004. Investigation of the differential response of near-isogenic lines of durum wheat (*Triticum turgidum* var *durum*) to cadmium. PhD thesis. Dept. of Biology, University of Western Ontario,London, Ontario.

Szmigielska, A.M., Van Rees, K.C.J., and Cieśliński, G. 2002. Gas chromatographic analysis of low molecular weight organic acids in roots and shoots of durum wheat plants. Communincation in Soil Science and Plant Analysis. 33: 1415-1423.

Taylor, G.J., Blamey, F.P.C., and Edwards, D.G. 1998. Antagonistic and synergisitic interactions between aluminium and manganese on growth of Vigna ungiculata at low ionic strength. Physiologia Plantarium. 104: 183-194.

Tester, M., and Leigh, R.A. 2001. Partitioning of nutrient transport processes in roots. Journal of Experimental Botany. 52: 445-457.

White, P.J., Bowen, H.C., Woolaway, K.E. 2000. Transport of calcium to the xylem in plant roots. Journal of Experimental Botany. 51: Supplement, 5.

Yeo, A.R., Yeo, M.E., and Flowers, T.J. 1987. The contribution of an apoplastic pathway to sodium uptake by rice roots in saline conditions. Journal of Experimental Botany. 38: 1141-1153.

Zenk, M.H. 1996. Heavy metal detoxification in higher plants- a review. Gene. 179: 21-30.

Zhao, F.J., Lombi, E., Breedon, T., and McGrath, S.P. 2000. Zinc hyperaccumulation and cellular distribution in *Arabidopsis halleri*. Plant, Cell and Environment.23; 507-514.

Zhao, Z.Q., Zhu, Y.G. Smith, S.E., Smith, F.A. 2005. Cadmium uptake by winter wheat seedlingsin response to interactions between phosphorus and zinc supply in soils. Journal of Plant Nutrition. 28: 1569-1580.

APPENDICES

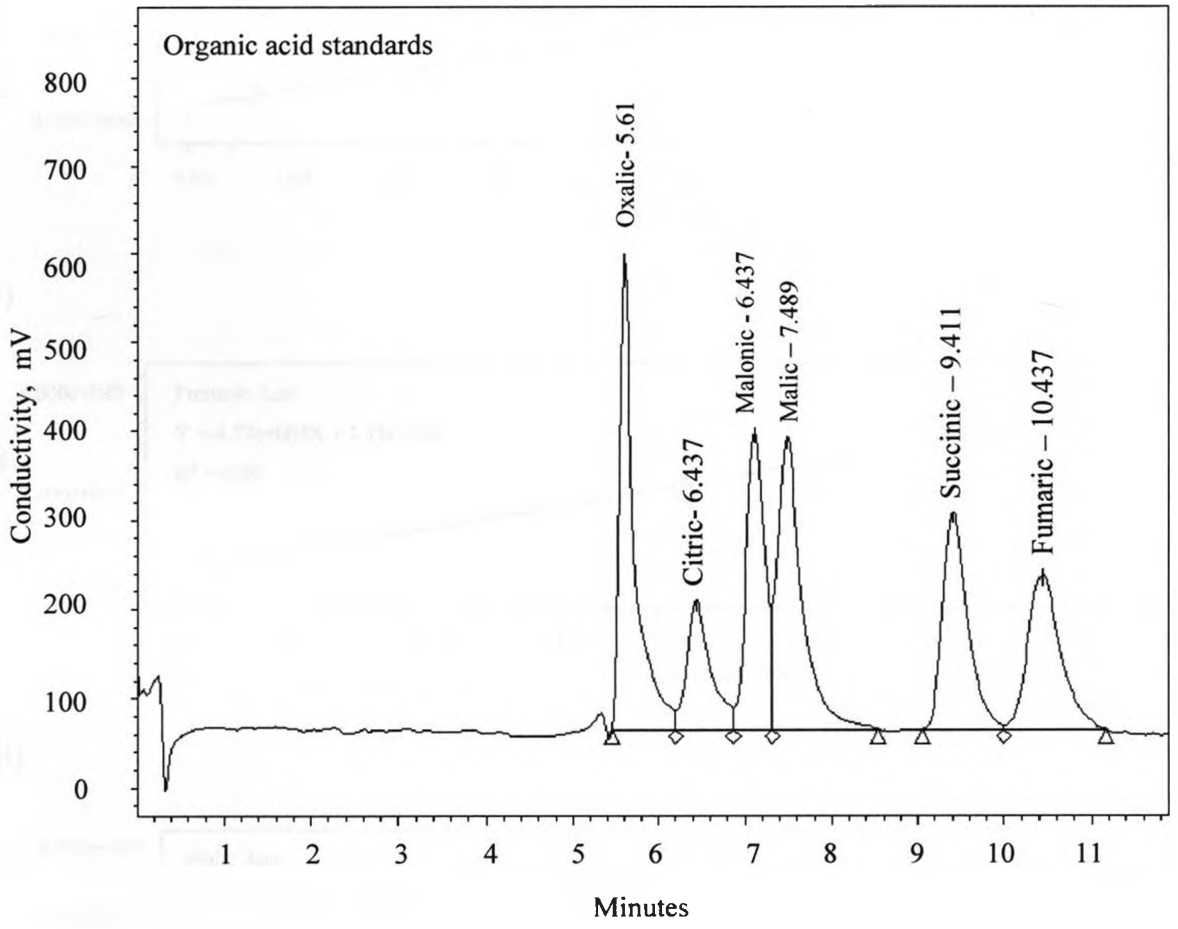
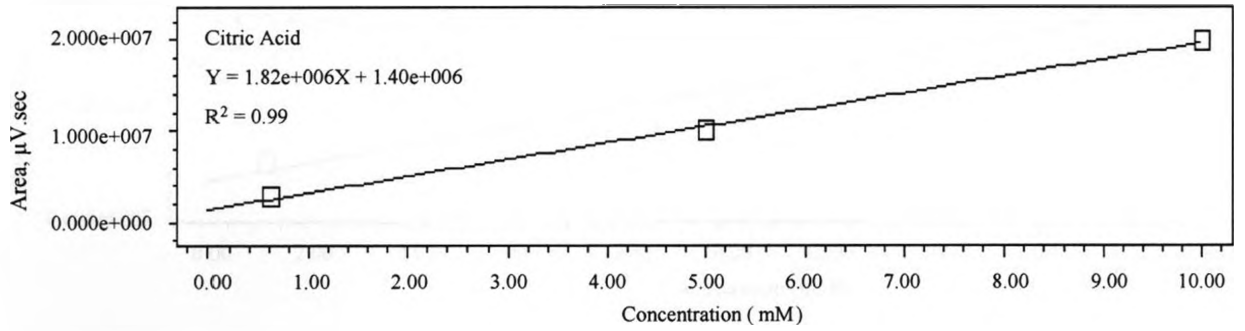
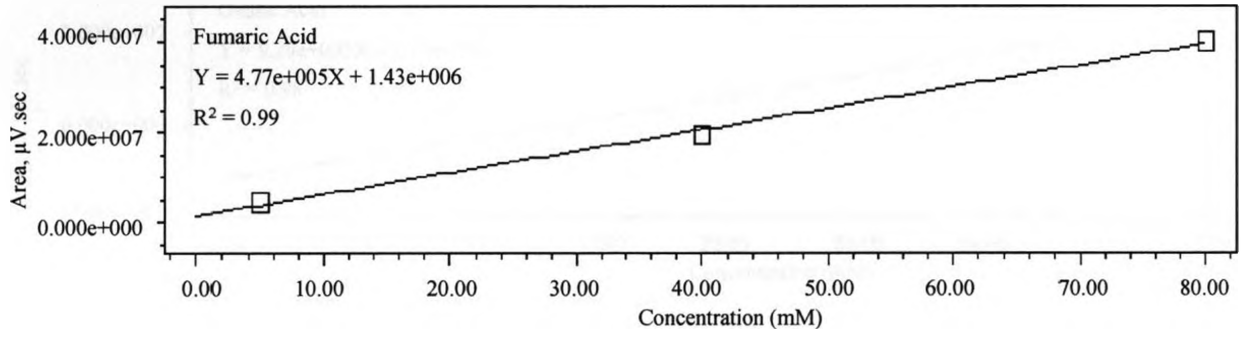


Figure A1: A typical chromatogram of six different organic acid standards. Organic acid standard mixtures were made in distilled water and assayed using the ion chromatography.

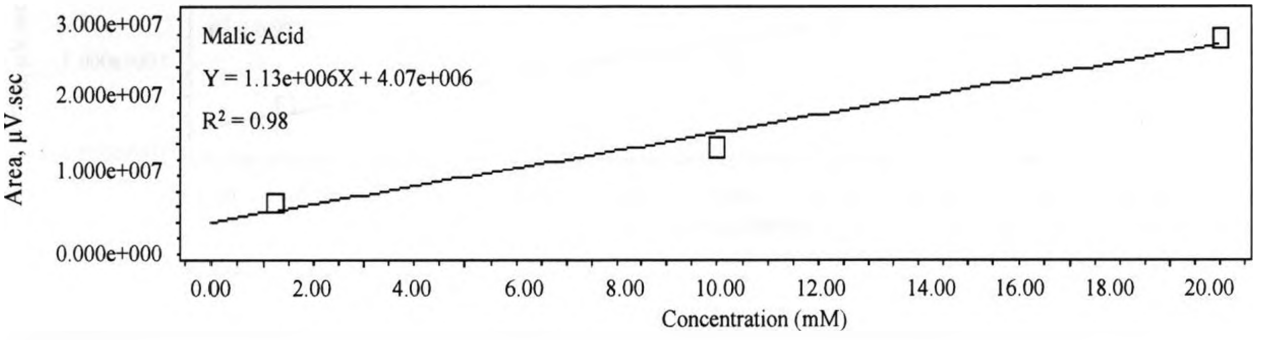
(i)


Citric Acid
Y = 1.82e+006X + 1.40e+006
R² = 0.99
Area, µV.sec
2.000e+007
1.000e+007
0.000e+000
0.00
1.00
2.00
3.00
4.00
5.00
6.00
7.00
8.00
9.00
10.00
Concentration (mM)

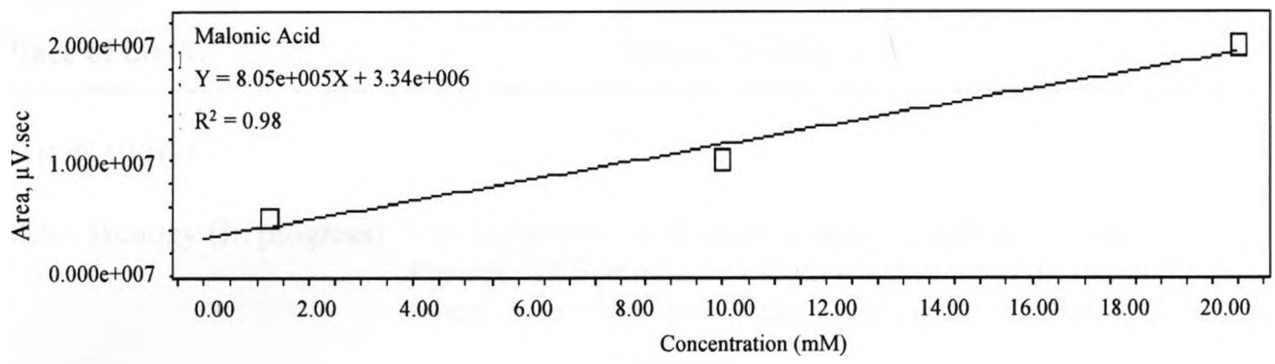
(ii)


Fumaric Acid
Y = 4.77e+005X + 1.43e+006
R² = 0.99
Area, µV.sec
4.000e+007
2.000e+007
0.000e+000
0.00
10.00
20.00
30.00
40.00
50.00
60.00
70.00
80.00
Concentration (mM)

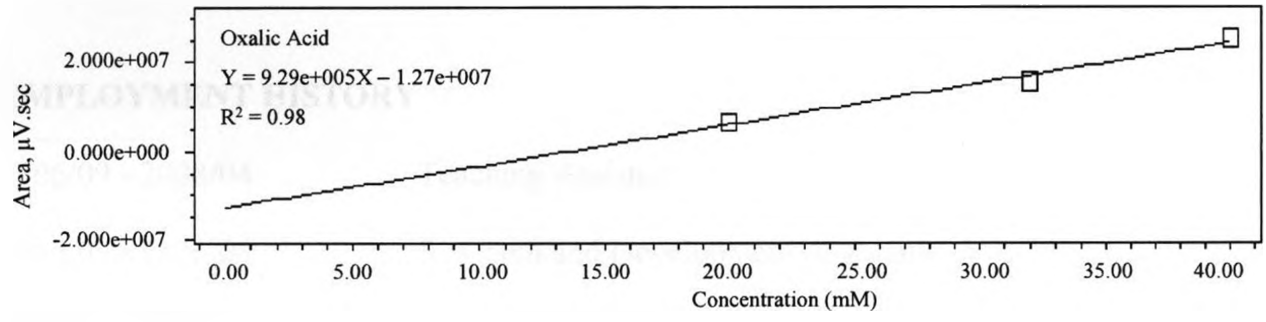
(iii)


Malic Acid
Y = 1.13e+006X + 4.07e+006
R² = 0.98
Area, µV.sec
3.000e+007
2.000e+007
1.000e+007
0.000e+000
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Concentration (mM)

(iv)



(v)



(vi)

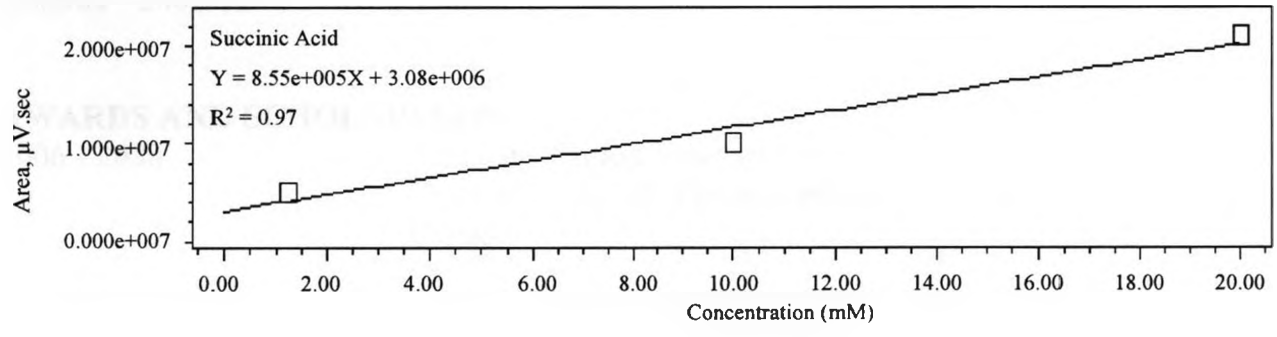


Figure A2: Calibration curves for different organic acid standards. Calibration curves for (i) citric acid (ii) fumaric acid (iii) malic acid (iv) malonic acid (v) oxalic acid and (vi) succinic acid were determined from the area under the peaks corresponding to different concentrations.