Selection of tomato genotypes that hyperaccumulate cadmium, for use in phytoremediation of cadmium contaminated sites

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DECLARATION

I hereby declare that the main text in this manuscript is an original work of mine, except where acknowledged, and no part of it has been previously submitted for the award of any other degree.

I also declare that, to the best of my knowledge any assistance I received in the experimentation presented, and all sources of information cited in this manuscript have been acknowledged.

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ABSTRACT

Cadmium (Cd) pollution results from industrialisation such as mining, waste water irrigation, electroplating and the use of phosphate fertilizers that contain traces of cadmium. A large proportion of semi-urban areas are contaminated with Cd due to intensive horticultural production that requires the use of phosphate fertilizers and effluent and sludge. Presence of high levels of Cd in these soils results in accumulation of Cd in the food crops. Consumption of Cd rich food items can induce cancer as well as causing health impacts in humans. Accumulation of Cd in the soils and plants can also cause other environmental impacts.

One way to minimise these adverse impacts would be to remove Cd from contaminated soils by chemical or biological means. Chemical removal of Cd from contaminated soils is very expensive. However, techniques such as phytoremediation may be used to remove Cd from contaminated soils economically. This technique however requires special types of plants that can grow well in Cd contaminated soils and also accumulate large quantities of Cd in their shoots.

The aim of this study was to select genotypes of tomato that would grow well in media containing high concentrations of Cd and accumulate large concentrations of Cd in their shoot tissues, so that they may be used in phytoremediation of Cd contaminated sites.

Seven concentrations (0, 10, 30, 100, 200, 500 and 1000 µM) of Cd were used in Murashige and Skoog (MS) tissue culture media, and the plants were grown in these media for 5 weeks. Seed germination was not affected by high Cd in most of the

genotypes. The seedling height increased at low Cd levels, but at higher levels of Cd (500 and 1000 µM), the heights were reduced in all genotypes. Ten of the 25 genotypes showed tolerance to high level of Cd. Shoot weight and leaf Cd concentrations were determined and the genotypes were classified as those having low Cd uptake (6 genotypes), medium Cd uptake (12 genotypes) and high Cd uptake (6 genotypes). The highest shoot Cd concentration was found in the genotypes Nash2 and Grosse Lisse and the lowest in Big Beef at 1000 µM. The highest Cd uptake was observed in Grosse Lisse and a wild tomato genotype CLt91t6D4 and the lowest uptake occurred in Oxheart.

Based on this *in vitro* culture experiment, four genotypes were chosen and were grown in a glasshouse for up to 9 weeks to test for their Cd accumulation patterns in sand culture. The plant height of Grosse Lisse was not affected by high Cd concentration (1000 µM) but the genotype Tiny Tom showed reduced shoot height. The higher Cd concentration and uptake was found in Big Beef after 3 weeks and 5 weeks and in Grosse Lisse after 5 weeks and 9 weeks. Based on this experiment, it was concluded that the genotypes Big Beef, Grosse Lisse and Burke's Backyard could be used in phytoremediation of Cd polluted (up to 500 µM Cd) soils.

The results also showed marked differences between plant tissue culture and sand culture for Cd uptake. The relative response of the genotypes in tissue culture experiment was similar to that found in sand culture. However, the leaf Cd concentrations were up to 4 times higher in *in vitro* cultured plants than in those grown in sand culture. This is possibly due to increased availability of Cd in tissue culture media. On the basis of these results, it was concluded that tissue culture technique can be used to rank tomato genotypes for Cd accumulation or tolerance, but the tissue culture conditions will result in overly high levels of Cd in their leaves.

A longer term (17 weeks) glasshouse experiment was also conducted using two genotypes of tomato to test for cadmium uptake and distribution amongst different parts of the shoots. The two genotypes were grown in sand culture and were treated with three concentrations (0, 100 and 500 µM) of Cd for 17 weeks. The high Cd treatment (500 µM) did not affect leaf number in Grosse Lisse. The plant height increased in Big Beef at 100 µM and 500 µM Cd when compared to control. The Cd treatment did not affect chlorophyll content of the two genotypes nor was any difference in foliar symptoms observed in aerial parts of either genotype. The results showed that the highest leaf Cd concentration was found in the genotype Grosse Lisse after 7 weeks and in Big Beef after 17 weeks at 500 µM Cd. Leaves accumulated more Cd than stems and the Cd concentrations in the stems increased over time. The results also indicated that the leaves of Grosse Lisse had higher Cd uptake than Big Beef at 500 µM Cd.

Near infrared spectroscopy based on effective wavelengths and chemometrics was proposed to discriminate and investigate the cadmium content in the stems of four tomato genotypes (Big Beef, Tiny Tom, Burke's Backyard and Grosse Lisse). Cadmium concentrations of the stem tissues were obtained by wet digestion and atomic absorption spectrometry. The AAS Cd readings were regressed against different near infrared spectral transformations using partial least squares regression methods. The NIR predicted tissue Cd concentration was highly correlated (R^2=0.9989) with the AAS measured Cd concentration. These results indicated that the use of NIR spectrometry technique could provide an accurate, reliable and non destructive method of predicting Cd concentrations in tomato tissues.

The genotypes that were used in NIR analysis were also tested for Cd concentration using X-ray fluorescence spectroscopy. The results showed that the Cd

concentrations of XRF spectroscopy correlated well with those measured via AAS (R^2=0.9811). Furthermore, the XRF data showed that an increase in tissue Cd concentrations in the stem tissues also increased the concentrations of other elements such as calcium, zinc, silicon, chlorine, sulphur, rubidium, potassium and phosphorus.

Both the NIR and XRF techniques provide to produce reliable results for detecting Cd concentrations in tomato tissues, and these techniques can be readily used to determine Cd concentrations in plants. These techniques will allow non destructive means of detecting metals in plant tissues and they are also more economical than AAS spectroscopy method.

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TO

MY LATE HUSBAND

&

MY LATE PARENTS

ABBREVIATIONS

AAS	Atomic Absorption Spectroscopy
Al	Aluminium
As	Arsenic
BAP	Benzylaminopurine
C	Celsius
Ca	Calcium
Cd	Cadmium
$CdSO_4$	Cadmium sulphate
Cl	Chlorine
Co	Cobalt
CPWS	Center for Plant & Water Science
Cr	Chromium
Cu	Copper
$CuSO_4$	Copper sulphate
Cvs	Cultivars
df	Degrees of freedom
FAO	Food and Agriculture Organisaion
Fe	Iron
g	Gram
g	Gravity
g/L	Gram per Litre
h	Hour
IAA	Indole-3-acetic acid
K	Potassium
L	Litre
LDW	Leaf dry weight
LSD	Least significance difference
Mg	Magnesium
min	minute
ml	Millilitre
mm	Millimetre

Mo	Molybdenum
MRI	Magnetic resonance imaging
MS medium	Murashige and Skoog's (1962) medium
MSC	Multiplicative scatter correction
MSW	Municipal solid waste
MT salt	Murashige and Tucker (1969) salt
NAA	1-naphthaleneacetic acid
Nb	Niobium
NIRS	Near-infrared spectroscopy
NMR	Nuclear magnetic resonance
NS	Not significant
NSW	New South Wales
P	Phosphorus
Pb	Lead
PCA	Principal Component Analysis
Pd	Palladium
PLSR	Partial least square regression
psi	Pounds per square inch
PTC	Plant tissue culture
R	Correlation coefficient of calibration
Rb	Rubidium
RO water	Reverse osmosis water
rpm	Revolutions per minute
RMSEP	Root mean square error of prediction
RMSECV	Root mean square error of cross validation
s	Second
S	Sulphur
SDW	Stem dry weight
SDR	Standard deviation ratio
SE	Standard error (of the mean)
SEP	Standard error of prediction
Si	Silicon
SNV	Standard normal variate
SPAD	Soil-Plant Analysis Development (SPAD)
SSC	Soluble solids content

SWNIRS	Short wavelength near infrared spectroscopy
VISNIR	Visible and near infrared spectroscopy
X-ray CT	X-ray computed tomography
Zn	Zinc
Zr	Zirconium

Chapter 1

Introduction

1.1 Cadmium

Cadmium (Cd) is one of the most toxic heavy metals and widely distributed in the environment (Zhang *et al.* 2009). The irrigation of waste water from industries and urban areas, the use of phosphate fertilizers and sewage sludge can cause Cd pollution in agricultural soils (Zhang *et al.* 2009). The presence of cadmium in the soil causes phytotoxicity via membrane distortion, reduction in transpiration, nutrient deficiency, necrosis, wilting, leaf curl and growth inhibition (Zhang *et al.* 2009). Distribution and contamination of Cd in soil is due to natural and man-made sources. In natural agricultural soil, Cd level in soil range from 0.1 to 0.5 mg/kg. Intrusive and extrusive rocks, foliated and non foliated rocks contained Cd from 0.02 to 0.2 mg/kg. Sedimentary rocks have high cadmium from 0.1 to 25 mg/kg. Natural gas, coal and oil contain Cd from 0.5 to 1.5 mg/kg but nitrogen phosphate and potash fertilizers contain Cd from 10 to 200 mg/kg (International Cadmium Association 2010). In Australia, Cd concentrations in soils range from less than 0.1 to 0.5 mg/kg (Vege notes, 2003). The roots of plants can easily absorb toxic metals from contaminated soils and metals will be translocated to aerial parts. This accumulation could affect food quality and safety (Arora, 2008). Consumption of Cd rich food can cause serious health problems such as immunological deficiencies, growth inhibition, disability, upper gastrointestinal cancer and renal tubular disease (Arora, 2008).

More than 400 species of land plants are known to hyperaccumulate heavy metals. These plants are found in restricted areas and are highly tolerant to metals. They also translocated metals which from roots accumulate in their shoots (Sun *et al.* 2006). Hyperaccumulator plants can take-up more than 10,000 mg/kg for Zn, more than 1000 mg/kg dry mass for Ni and more than 100 mg/kg for Cd (Baker *et al*. 1994; Brown *et al.* 1994). Cadmium moves rapidly in the soil and it affects human health (Sun *et al.* 2006). The absorption and translocation of nutrients from roots to shoots is influenced by the presence of heavy metals in the soil solution (Moral *et al.* 2002). This contaminated metal can however be removed by phytoremediation technique.

1.2 Phytoremediation

Phytoremediation is the use of hyperaccumulating plants for removing toxic metals from hazardous waste sites and metal polluted soil environment (Chehregani *et al.* 2009). This method is effective for use in contaminated areas. *Thlaspi caerulescens* has high ability to absorb cadmium from soil and accumulate in their shoots (Ishikawa *et al.* 2006). *Brassica juncea* can extract Cd from polluted soil and it still exhibits regular plant growth (Ishikawa *et al.* 2006). Phytoremediation technology is less costly than conventional methods (Free Essays, 2003).

1.3 Phytoextraction

Phytoextraction is the use of metal accumulating plants to remove metals from contaminated soil. Plants that can take up high amounts of metals are grown in contaminated soil. Upper parts of plants accumulate metals in large concentrations. These plants can be harvested and removed from metal contaminant sites. Success of phytoextraction depends on the degree of soil pollution, type of metal, ability of the plant to absorb and distribute their metals in to the shoot systems (Lasat, 2002).

1.4 Health

Humans are exposed to toxic metals (Cd, Pb, Zn, Ni, Hg, As and Cu) through various means such as inhalation of contaminated air and daily intake of metal containing foods that could cause serious health problems (Li *et al*. 2007). Some vegetable and cereal crops such as parsley, celery, lettuce, cabbage, spinach, sweet potatoes, carrot, beetroot, oats and soybeans can contain Cd from (30-150 ppb). The Cd content of meat and fish is lower (5-40 ppb) than internal organs of animals (>1000 ppb) (International Cadmium Association 2010).

1.5 Cadmium toxicity to plants

The hyperaccumulation of Cd in plants causes growth retardation and mortality due to reduction in water and nutrient absorption, chlorophyll synthesis, gaseous exchange and transpiration (Cho, 2004). Toxicity symptoms in plants include chlorosis, necrosis, leaf roll, change of cell membrane and oxidative stress (Meng *et al.* 2009). Contaminated soil solutions contain 0.32 to 1 mM Cd but uncontaminated soil solutions contain 0.04 to 0.32 mM Cd (Benavides *et al.* 2005).

In tomato cultivar Micro Tom, leaf and fruit growth was inhibited when the plants were treated with 1000 µM Cd (Grato *et al.* 2008). Most of the shoots were dead in apple tree rootstocks M9 and B396 that were treated with 500 to 2000 µM $CdSO_4$ (Sakalauskaite *et al.* 2006). The shoot and root weight decreased in *Sedum alfredii* at 200 to 800 µM Cd (Yang *et al.* 2004). The shoot and root lengths of tomato cv. Seokwang decreased at 100 µM Cd as compared to control after 9 days (Cho, 2004). The fresh weight of sugarcane decreased when treated with 500 µM Cd but no mortality was observed at 500 µM (Sereno *et al.* 2007). The number of leaves, root length and shoot length decreased in tomato at 10 µM Cd compared to control after

12 days (Deef, 2008). Leaf, stem and root weight of tomato cv. Hezuo 903 and Jiang-shu 14 decreased at 10 µM Cd compared to control (Dong *et al.* 2005). The leaf and root weight decreased in tomato treated with 50 µM Cd compared to control (Quariti *et al.* 1997). Shoots weight, root weight and leaf area of tomato decreased at 100 µM Cd compared to control (Djebali *et al.* 2005). The fresh weight and dry weight of leaf and stem decreased in tomato cultivar Tres cantos treated with 10 µM and 100 µM Cd (Lopez-Millan, 2009). The fresh weight of root and shoot decreased in pea treated with 30 µM Cd compared to control (Lima *et al.* 2006). Root and shoot dry weight of barley decreased at 5 µM Cd compared to control (Wu *et al.* 2004). Root and shoot weight of *Solanum nigrum* and *Solanum melanogena* decreased treated with 100 µg/g Cd compared to control (Sun *et al.* 2006). Leaf dry weight of lettuce and spinach decreased at 0.32 µM Cd compared to control (McKenna *et al.* 1993). Shoot dry weight of *Thlaspi caerulescens* increased treated with 10 µM Cd but decreased at 20 µM and 50 µM Cd (Keller *et al.* 2006). Leaves, stems, roots and bulbs weights of *Arum* decreased at 10 and 50 µM Cd compared to control. Root and shoot weight of radish decreased at 10 µM Cd and leaf, stem and root weight of water spinach decreased at 10 µM Cd compared to control (Kashem *et al.* 2008).

Azevedo *et al.* (2005) reported that sunflower grown in *in vitro* and treated with 500 µM increased Cd content of calli and shoots. Chlorosis symptoms were found in tomato leaves at 10 µM Cd and necrotic spots were found at 100 µM Cd (Lopez-Millan *et al.* 2009). The chlorophyll content, fresh weight and dry weight of tomato was reduced when they were treated with 7120 µM Cd compared to control (Cherian *et al.* 2007). The cadmium content and Cd accumulation increased in the shoots of two rice cultivars at 5.0 µM as compared to those that were treated with 1.0 µM

(Zhang *et al.* 2009). The shoot weight increased in tomato plants that were treated with 1000 µM Cd and inoculated with *Methylobacterium oryzae* strain compared to control (Madhaiyan *et al.* 2007). Cadmium reduced fresh weight and dry weight of roots and shoots in barley at 25 µM Cd and when inoculated with salicylic acid (Metwally *et al.* 2003).

1.6 Tomato

Tomato is one of the most popular vegetable crops in the world and is grown in glasshouses, net houses and in the field. Tomato fruits provide nutrients for humans and fresh fruit can be used to produce pastes and ketch up. Tomato contains vitamin A and C, fiber, minerals and is cholesterol free (Mohamed *et al*. 2010). Tomato also contains carotenoids and lycopene that possess antioxidant properties. Thus, consumption of tomato can protect prostate, breast, colorectal, pancreatic and lung cancer (George Mateljan Foundation, 2009). The tomato belongs to the Solanaceae family which includes potatoes, capsicums and eggplants. In the tropics, *Lycopersicon esculentum* is a perennial plant but in northern climates it is grown as an annual. Peru, Bolivia and Ecuador area of the Andes Mountains are the native lands of tomato (Prince Edward Island, 2005). Multiplication of tomato explants occur from shoot tips, cotyledons, leaf, callus and hypocotyls (Sheeja *et al.* 2004). Successful plant regeneration and embryo induction from *in vitro* cultures are fundamentally dominated by the genotype and physiological status of the explants (Sheeja *et al.* 2004).

The aim of this research was to select cadmium hyperaccumulating tomato genotypes and to compare plant growth rates at different concentrations of cadmium. The specific objectives include:

1. Optimise tissue culture conditions for screening tomato genotypes
2. Screen a wide range of tomato genotypes for their ability to accumulate high concentrations of cadmium in their shoots
3. Determine responses of four genotypes of tomato to cadmium in sand culture in a glasshouse
4. Test long term effects of exposing tomato to Cd in sand culture in a glasshouse
5. Develop rapid method of detecting cadmium concentrations in the tissues using NIR and X-ray fluorescence spectroscopy.

Please refer to Figure 1 for various studies undertaken and the interrelationships between them.

Chapter 1 includes general introduction to cadmium, Cd contaminated sites and about tomato and its importance.

Chapter 2 reviews current literature on various aspects of phytoremediation and tissue culture and identifies the gaps in literature in this field.

Chapter 3 describes general methods used in media preparation and chemical analysis of plant samples.

Chapter 4 deals with optimising tissue culture conditions such as the media type, culture tubes, number of seeds, harvesting time and response of various explants to tissue culture.

Chapter 5 reports results of a large experiment conducted in tissue culture using 25 genotypes of tomato that were exposed to 7 concentrations of cadmium.

Chapter 6 validates tissue culture results by examining plant response to Cd in sand culture and in a glasshouse. The Cd concentrations of this experiment are also compared with those of tissue culture.

Chapter 7 examines long term effect of exposing tomato plants to Cd in sand culture in terms of Cd uptake and distribution in various shoot tissues.

Chapter 8 tests the use of non-destructive methods (NIR and XRF) of determining Cd concentrations in tomato tissues by verifying the values of these techniques with those obtained from atomic absorption spectroscopy (wet digestion method).

Chapter 9 includes general discussion and

Chapter 10 summarises the results of all the experiments carried out, and also identifies future research needs.

The Appendices section includes the graphs showing the relationships between leaf Cd concentrations and leaf dry weight (Appendix 1) and Cd uptake (Appendix 2) by 24 genotypes of tomato grown in tissue culture at various concentrations of cadmium. These graphs will help select suitable genotypes of tomato for use in phytoremediation of Cd contaminated soils.

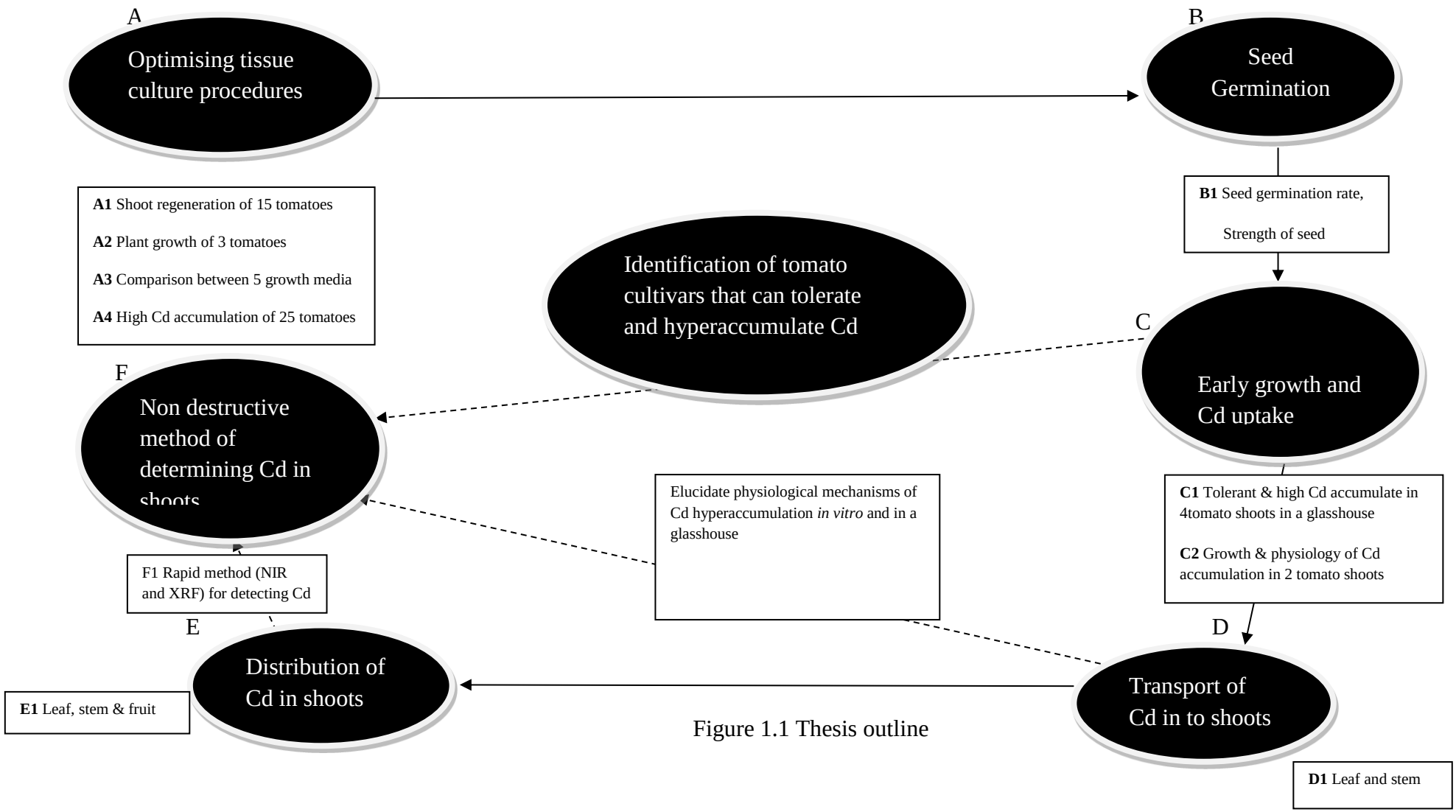


Figure 1.1 Thesis outline

Chapter 2

Review of Literature

2.1 Seed germination

Seed germination is an essential event in plant development and is highly influenced by the environment (Islam, 2009). Optimum seed germination depends on suitable temperature, humidity and moisture content (Burgos *et al.* 2008). Pre-sowing treatment which includes sowing seeds in water over night produced the highest germination percentage. Pre-treatment allows the seed, to absorb water partially resulting in higher germination percentage when compared to untreated seed (Islam *et al.* 2009).

2.2 Effects of Cd on seed germination

Seed germination was inhibited and the fresh weight of roots and shoots decreased in Mediterranean leguminous shrub *Dorycnium pentaphyllum* treated with 1000 µM $CdCl_2$ compared to control (Lefevre *et al.* 2009). Seed germination, seedling height, root length and seedling dry weight was decreased in *Leucaena leucocephala* at 100 mg/kg lead nitrate and cadmium sulphate as compared to control (Shafiq *et al*. 2008). Seed germination was inhibited in mustard (*Sinapsis arvensis*) at 300 µM Cd and 150 µM Pb as compared to control (Heidari and Sarani 2011). Seed germination decreased in Zinnia plant at 50 µM $CdCl_2$ as compared to control (Thamayanthi *et al*. 2011). Seed germination, root, shoot and seedling length and dry weight were decreased in *Albizia lebbeck* at 10, 30, 50, 70 and 90 µM Cd and Pb as compared to control (Farooqi *et al*. 2009). Seed germination percentage decreased in cowpea (*Vigna unguiculata*) at 40, 80 and 160 µM $CdCl_2$ (Rumaih *et al*. 2001).

2.3 Tissue culture

Tissue culture technique is essential for shoot regeneration and mass production of plants in a short period without seasonal disturbance (Sharry and Teixeira da Silva 2006). Genetic engineering methods assist in the development of virus and fungi disease free cultivars and multiplication of high value cultivars for agronomic market requires tissue culture (Bhatia *et al*. 2004). Seeds of tomato were cultured in MS basal medium supplemented with different concentrations of activated charcoal, ascorbic acid and casein hydrolysate. The results showed that the medium containing 5 mg L^{-1} charcoal reduced shoot response (47%) as compared to control (69%). Shoot height of tomato increased at 10 mg L^{-1} charcoal but decreased at 60 µM Ascorbic acid. Callus production was reduced at 100, 200, 500 and 1000 mgL^{-1} Casein hydrolysate (Bhatia and Ashwath 2008). The MS medium supplemented with 17.8 µM BA produced maximum number of shoots *Casuarina* hybrid (Shen *et al*. 2009).

Phalaenopsis orchid plantlets were cultured in sugar free MS basal medium treated with different concentrations of uniconazole. Photosynthetic pigments and total carotenoids increased at 6.86 µM uniconazole as compared to control (Cha-um *et al*. 2009). The seeds of eggplant were cultured in MS basal medium containing thidiazuron (TDZ) and benzyladenine (BA). The medium containing 0.45 µM TDZ and 13.3 µM BA produced maximum number of shoot buds per explants and callii compared to control (Franklin *et al*. 2004). Seeds of Turkish cowpea were cultured in different MS media and supplemented with plant growth regulators. The medium with 3.33 µM BAP produced maximum callus diameter and higher number of shoot per plant compared to control (Assim *et al*. 2009). The explants of water chestnut

were cultured in MS medium supplemented with growth regulators. Eighty percent of the callus grew rapidly and regenerated several times when supplemented with 4.44 µM BA and 11.31 µM 2, 4 D compared with other combination of growth regulators. The highest shoots number per explant and shoot length were observed in treatment supplemented with 4.88 µM BA and 2.69 µM NAA (Hoque *et al*. 2006).

The ornamental prickly pear cactus was propagated by micropropagation using different concentrations of cytokinin and carbohydrates. The MS (Murashige and Skoog 1962) media supplemented with BA (N6-Benzyladenine) increased the number of shoots compared to DAP (dimethylaminopurine) and K (6-furfurylaminopurine). The shoot length and shoot fresh weight increased in media supplemented with 7.5% sucrose compared to 10% sucrose. The medium with 50% MS increased shoot growth compared to 100% MS medium (Estrada-Luna *et al*. 2008). The leaf explants derived from *in vitro* seedlings of 2 cultivars of sweet orange (*Citrus sinensis*) were grown in MT salts (Murashige and Tucker 1969), 0.5 mg/L BA, 0.5 mg/L Kinentin and 0.1 mg/L NAA. Highly significant differences of shoot bud regeneration were observed between the 2 cultivars (Khan *et al*. 2009). The shoot formation from leaf explants of almond was monitored. Medium which was supplemented with 2,4D (2,4-dichlorophenoxyacetic acid) and NAA (naphathaleneacetic acid) increased the production of nodular callus. Plant growth regulator affected adventitious bud formation and frequency of shoot regeneration. Adventitious bud formation increased in media supplemented with 4.9-24.6 µM IBA and 5.4-10.7 µM NAA (Ainsley *et al*. 2000).

2.4 MS medium with cadmium

The effects of cadmium phytotoxicity in plants include retardation of growth and transpiration, damage of tissues, instability of lipid composition, enzymatic stress, oxidative stress and mortality (Shekhawat *et al*. 2010). *In vitro* techniques can be applied for selection of metal resistant plants (Gatti 2008). Cell lines of tomato were cultured in MS medium supplemented with growth regulator and added with different Cd concentrations. The medium with 200 µM Cd decreased fresh weight (37%) and dry weight (54%) of tomato compared to control. Plant growth was reduced at 1000 µM Cd after 14 days (Huang *et al.* 1987). Two cultivars of potato plantlets (Asterix and Macaca) were micropropagated in MS medium treated with different Cd concentrations. The root dry weight of Asterix was reduced when treated with 100 µM Cd but shoot dry weight was increased. The root and shoot dry weight of Macaca increased at 100 µM, 200 µM and 400 µM Cd when compared to Asterix (Goncalves *et al*. 2009). The callus of *Solanum nigrum* was cultured in MS medium supplemented with different concentrations of $CdSO_4$ with proline pre-treatment. The growth of callus was inhibited at 150 µM Cd. Proline accumulation decreased in callus at 100 µM Cd. The shoots of *Solanum* increased and rooting rate was up to 1.19 times and root length up to 1.47 times when treated with 30 µM Cd as compared to control. The cellular Cd content increased 16.8%, 15.6% and 9% when treated with 50,100, 150 µM Cd respectively (Xu *et al*. 2009). The callus of *Brassica juncea* was cultured in MS medium supplemented with growth regulators and $CdSO_4$. The media containing 200 µM Cd proved to be lethal to callus growth. Fifty percent of hypocotyls were formed at 40 µM Cd compared to control in *Brassica juncea* (Nehnevajova *et al*. 2007). Tomato callus was cultured in MS medium containing different concentrations of $CdCl_2$. The reduction in growth rate and

appearance of reddish brown patches were observed in callus treated with 100, 150 and 200 µM $CdCl_2$. Callus treated with 200 µM Cd showed 73.6% reduction in tolerance compared to control (Shekhawat *et al*. 2010). Two apple-tree rootstocks M.9 and B.396 were cultured in MS medium and supplemented with different concentrations of $CdSO_4$. The micro shoot growth decreased and total growth inhibited at 200-2000 µM in M.9 and B.396 cultivars compared to control. Almost all micro shoots were dead in both cultivars at 500-2000 µM $CdSO_4$ compared to control. Chlorophyll contents and carotenoids decreased with increasing $CdSO_4$ concentrations in leaves of both cultivars when compared to control (Sakalauskaite *et al.* 2006).

2.5 Acclimation of tissue cultured plants

Little is known about adaptation of micropropagated plantlets and transplants to field condition (Donnelly *et al.* 1985). Red raspberry plantlets incubated at low light intensity (25 $\mu Es^{-1}m^{-2}$) can be transplanted successfully in field conditions as they had greater pigment content and smaller leaf size when compared to high light intensity cultured plants (120 and 160 $\mu Es^{-1}m^{-2}$) (Donnelly *et al.* 1985). Improvement of acid soil tolerance in *Sorghum* has been achieved via micropropagation method where by tolerant lines have been produced from sensitive parental lines (Miller *et al*. 1992).

2.6 Effects of cadmium in soil culture in glasshouse

Cadmium is a non-essential element for plant growth and is a toxic pollutant in the soil environment (Sorial and Abd El-fattah 2001). Cadmium is highly mobile in the soils and it is easily absorbed by plant roots and then translocated to edible parts of the plant to cause health risks to humans (Jin-Tian *et al*. 2010). Smelting ore by

mining companies, carbon emission, application of Cd containing phosphate fertilizers and organic manure in agriculture can cause metal contamination in soils. Cadmium has high solubility in soil and is active at low concentrations. It accumulates in living organisms and can cause cytogenic, mutagenic and carcinogenic effects (Kuriakose and Prasad 2008).

Cadmium can cause inhibition of seed germination and reduction in growth and development, photosynthetic rate and water and nutrient uptake (Lopez-Millan *et al.* 2009). It can also cause membrane damage, leading to interaction with nucleic acids (Lopez-Millan *et al*. 2009). Seeds of soybean were grown in soil culture in a greenhouse. Forty percent of plant height and 34% of dry weight was reduced at 890 µM Cd in soybean when compared to control (Shute and Macfie 2006). Seeds of tomato Micro Tom were sown in soil culture in a glasshouse. Micro Tom plants treated with 1000 µM Cd inhibited growth rate and accumulated cadmium in their leaves, fruits and roots after 20 days of exposure to Cd (Grato *et al*. 2008). Seeds of *Solanum nigrum* were sown in pots containing soil in a greenhouse. The results showed that the shoot weight was reduced by 55% and in root weight by 57% in the seedling stage when treated with 264 µM Cd. In mature stage reduction was 40% in shoots and 39% in the roots (Wang *et al*. 2008).

Seeds of corn and sunflower were sown in pots containing soil in a greenhouse. Fresh weight and dry weight of shoots decreased in corn and sunflower between 43% and 62% at 1779 µM Cd when compared to the control. Leaf area and chlorophyll content reduced in both corn and sunflower at 1779 µM Cd as compared to control. Sunflower plants had higher Cd concentration in roots and lower Cd in shoots compared to corn (Prista *et al*. 2008). Tomato seeds were grown in plastic

pots containing soil exposed to different temperatures in a greenhouse. Low soil temperature 45ºF inhibited seed germination. Soil temperature 75ºF to 85ºF increased seed germination and the leaves were green and normal. Higher roots temperature (85ºF) increased plant height and dry weight (Jaworski and Valli 1964). The seeds of tomato and sweet corn were planted in pots containing soil that was treated with different concentrations $CdCl_2$ in the glasshouse. The 1.75 µM $CdCl_2$ showed no significant effect on root dry weight of tomato but it decreased in corn root growth. Shoot dry weight reduced in tomato by 47% and in corn by 60% when treated with 1.75 µM $CdCl_2$ (Sameni *et al*. 1987). Pak Choi, tomato and radish were sown in soil treated with various concentrations of $Cd(NO_3)_2$ in a greenhouse. The treatment of 29.61µM $Cd(NO_3)_2$ had no significant effect on shoot growth in all vegetable crops. Root dry weight decreased in radish at 27 µM Cd. The accumulation of Cd in Pak Choi and tomato decreased in the order root> shoot> fruit but the order was shoot> root for radish (Shentu *et al*. 2008). Tobacco seeds were sown in a box containing soil in the glasshouse and the soil was treated with different concentrations of $CdCl_2$. The number of leaves decreased in tobacco cultivar at 1644 µM Cd. The uptake of cadmium increased in tobacco treated with 1644 µM Cd (Vasiliadou and Dordas 2009).

2.7 Effects of cadmium in sand culture in glasshouse

Cadmium is one of the most toxic heavy metals and it causes phytotoxicity to plants. High Cd accumulating plants show reduced in photosynthesis rate, diminished water and nutrient uptake, leaf chlorosis and necrosis (Haider *et al*. 2007). In a greenhouse experiment using sand culture, plant height, root weight and shoot weight of soybean were reduced at (1.0 µM) Cd at a pH of 4. The same Cd concentration (1.0 µM) and

low pH reduced chlorophyll content, photosynthetic rate, nutrient uptake and stomatal conductance (Haider *et al*. 2007). Tomato seedlings were sown in pots containing sand and were treated with the highest Cd concentration (7120 µM). This treatment reduced fresh and dry weight and chlorophyll content when compared to control (Cherian *et al*. 2007). Chlorophyll content, and shoot and root growth was decreased in pea plant treated with 534 µM Cd in sand culture (Sorial and Abd El-Fattah 2001). Barley plants were grown in sand culture in a glasshouse. High Cd treatment (7500 µM) inhibited root dry mass, reduced leaf gas exchange and photosynthetic pigments content (Vassilev *et al*. 2004). Garden pea was screened for tolerance to cadmium toxicity in sand culture in a greenhouse. The majority of pea genotypes was not tolerant and was dead when treated with 71 µM Cd after 10 and 15 days of exposure to Cd. The cadmium tolerance index varied significantly between pea genotypes from 35% to 90% and from 54% to 100% at Cd concentrations of 38 µM and 16 µM respectively. Shoots of pea genotypes were tolerant to high Cd concentration 38 µM (Belimov *et al.* 2003). Four switch grass cultivars were grown in sand culture in a temperature controlled greenhouse. Cadmium at 77 µM was phytotoxic to switch grass and Cd accumulated in all plant parts. Biomass accumulation decreased by 38% at 77 µM Cd as compared to control in switch grass. Biomass reduced in the order of leaf blade 39%, stem 47% and root 31% when treated with Cd 77 µM in switch grass. The highest Cd concentrations were found in root tissues (Reed *et al.* 1999).

Two soybean cultivars were grown in a greenhouse in sand culture and were tested for Cd accumulation. Soybean plants treated with 8.89 µM Cd showed 10 times higher Cd concentrations in roots than in shoots. Growth stage V2 contained 85% of Cd which was higher than that found at growth stage VC 68% in both parts of the

two soybean cultivars (Oliveira *et al*. 1994). Cadmium concentration (10.99 µM) caused moderate chlorosis in 45% and 60% of the leaves of split-root and intact plants respectively, as compared to control. The treatment of Cd (10.99 µM) increased Cd content in leaf, stem and root of both intact plants and split-root of tomato (Smith and Brennan 1983). Seeds of *Thlaspi caerulescens* were grown in sand culture in a glasshouse using four populations. The treatment of $CdSO_4$ (30 µM) reduced growth rate all populations when compared to control after 31 days of exposure to Cd. The Prayon population was more tolerant than the other four populations that were treated with 30 µM Cd (Roosens *et al*. 2003).

Seeds of two tomato cultivars (Hezuo 903 and Jiangshu 14) were grown in sand culture in a glasshouse. After 33 days, the two tomato cultivars treated with 10 µM Cd decreased plant height and leaf number by 48.9% and 31.5% respectively when compared to control. Root length and volume decreased in the two cultivars at 10 µM Cd after 33 days of exposure. Leaf, stem and root dry weight decreased by 69.8%, 76.2% and 68.3% respectively at 10 µM Cd in two tomato cultivars. Chlorophyll content was reduced in cultivar Hezuo 903 and Jiangshu 14 (52.8% and 41.6%) at 10 µM Cd compared to control. The treatment with 10 µM cadmium showed severe phytotoxicity symptoms (such as necrotic patches with browning of root system and leaf necrosis) than treatment with 1 µM Cd (Jing *et al.* 2005). Seeds of *Pisum sativum* were grown in sand culture in a greenhouse. The root and shoot biomass reduced in pea plants at the highest cadmium (30 µM). The root and shoot lengths were shorter at 30µM Cd than in control pots (Lima *et al*. 2006).

2.8 Cadmium in contaminated soils

Heavy metals are the major environmental contaminants and they pose ecological, evolutional and environmental problems. Heavy metals are present in the atmosphere, soil and water they can cause toxicity problems to all organisms (Benavides *et al*. 2005). Cadmium hyperaccumulating plants show reduced respiration, photosynthesis, plant-water relationships, nitrogen metabolism and even plant death (Liu *et al*. 2008). Neurotoxicity, hepatotoxicity and nephrotoxicity are caused by heavy metals (Benavides *et al*. 2005). The use of green plants that hyperaccumulate metals are used in phytoremediation of metal contaminated soils. This technique is low cost and environmentally sustainable (Yang *et al*. 2004). Phytoremediation technique has been found to be the most successful for cadmium polluted soils (Ishikawa *et al.* 2006).

Field experiment with *Lycopersicon esculentum* was established in a polluted soil and phosphogypsum amendment was used. The effect of phosphogypsum amendment was not significant when compared to un-amendment plot. Cadmium concentrations of tomatoes were found higher than other areas (Abril *et al*. 2008). Twelve samples of leafy vegetables and non leafy vegetables were grown in two different rural and urban areas. The levels of heavy metals obtained from different kinds of vegetables was Cd< Pb< Ni< Cu< Zn. The levels of Cd in all vegetable samples collected from the urban area were higher than those obtained from the rural area because urban area is affected by municipal, domestic, traffic and some industrial discharges (Demirezen and Aksoy 2006). Five Swiss populations of *Thlaspi caerulescens* were grown in the field. Soils were collected from Alps and prealps of Cd contaminated soils. They were compared with those of Ganges

population from France and Prayon population from Belgium. Soils were collected from metalliferous soils in mining areas. The Cd concentrations were significantly different between these populations. In general, the presence of 10 µM Cd decreased dry weight and increased Cd content in the shoots. The shoot dry weight was more tolerant to Cd than root dry weight and total root length. The Swiss population was similar to Ganges population but different from Prayon population (Keller *et al*. 2006).

Over all, heavy metals in soils derived from natural and anthropogenic sources and they caused plant toxicity, ion interaction in soil, nutrient imbalances and pH disorders. In agricultural soils, waste water irrigation caused excessive accumulation of heavy metals resulting in soil contamination. This accumulation could affect food quality and safety. Most plants are susceptible to even low Cd concentrations which inhibit the photosynthesis rate, reduce uptake and translocation of macronutrients and micronutrients. Cadmium phytotoxicity causes inhibition of plant growth and transpiration, damage of tissues, reduction of nutrient and water uptake, enzymatic stress, oxidative stress, leaf chlorosis and necrosis and mortality. Cadmium also affects the seed germination percentage, seedling length and seedling growth, and reduction in callus formation. Suitable temperature, humidity and moisture content are the major factors for seed germination. The micropropagation technique might provide large numbers of plantlets for further cultivation in metal polluted sites with or without using plant growth regulators. *In vitro* techniques can be applied for selection of metal resistant plant. The application of industrial effluents for irrigation in horticultural farms can result in accumulation of these metals in soils. The use of cadmium containing phosphate fertilizers and pest control chemicals in crop production may lead to serious health problems in humans due to accumulation of

Cd in the plants that would subsequently be consumed by humans. Phytoremediation is the use of hyperaccumulating plants for removing toxic metals from metal polluted soil environment.

Studies in various crops show differing responses of plant species to cadmium. These responses also differ from tissue culture to glasshouses and to field conditions. A large number of studies demonstrate that the genotypic variations in Cd tolerance can easily picked up in tissue culture studies. Based on these studies and the range of Cd concentrations used in testing the plant species for Cd tolerance, the following aims and objectives were formulated for the study.

Chapter 3

General Materials and Methods

3.1 Preparation agar medium

The laminar flow cabinet room was sterilised with ultra violet light for one hour. All equipment was rinsed with distilled water and automatic media dispenser was cleaned and the settings were checked to dispense required quantities of media. Murashige & Skoog (MS) Murashige & Skoog, 1962, Duchefa Scientific Pty. Ltd); 4.32 g/L was weighed and was added to conical flask containing 800 ml of distilled water. The conical flask was placed on a hot plate and the solution was stirred using a magnetic stirrer. When the solution was turned warm, 8 g/L of agar (Duchefa Scientific Pty.Ltd) and 30 g/L of sucrose were added and were allowed to mix thoroughly until all the salts were dissolved. The flask volume was adjusted to 950 ml and the pH of the solution was adjusted to 5.7 with sodium hydroxide or hydrochloric acid. The final volume of the flask was adjusted to 1000 ml. Once all the salts were dissolved, the solution was poured manually into tissue culture tubes (large tubes) or it was dispensed into small tubes using media dispenser (Jencons Scientific Ltd. Leighton Bezzard, England) which was set at 5, 10, 50 or 100 ml.

The tissue culture tubes were closed loosely with the lids and were transferred to the autoclave (Cominox, Model Stericlave 24S, Brianza, Italy). The media were autoclaved at 121ºC and 103 kPa for 21 minutes.

3.2 Preparation of slant agar medium

The 100 ml of the agar media (see Section 3.1) was mechanically dispensed into large tissue culture tube (150 mm x 70 mm, polypropylene, non-sterile, Sigma

Aldrich Pty.Ltd) and were autoclaved at 121°C and 103 kPa for 20 min. After sterilising, the lids were tightened and the tubes were placed on a bench in a slanting position and they were allowed to cool in that position. On cooling these tubes showed condensation of water vapours. Half of the tubes were left as is and the remaining half were transferred to laminar flow cabinet where in the condensed water was wiped with sterile tissue. Another lot of media was poured straight into tubes and were placed on the bench after autoclaving. This resulted in three types of media, viz., tubes with flat surface, tubes with slanting surface and the tubes with slanting surface, but was wiped free of condensation.

3.3 Preparation of potting medium for *in vitro* culture

Potting mix was purchased from Bunning's Warehouse and was dried in an oven for 24 hours at 60ºC. The dried media was sieved through to a 2 mm mesh sieve and the <2 mm fraction was used in the experiment. Into one litre conical flask, 800 ml of distilled water was added and heated on a hot plate. MS salts (4.32 g/L) were then added to warm water. The volume of the conical flask was made up to 1000 ml and the pH of the solution was adjusted to 5.77. Dried potting mix (26 g) was added to each of the large tissue culture plastic tube (150 mm x 70 mm) and then 25 ml of MS solution thus prepared as above was poured. The tubes were then autoclaved at 121ºC and 103 kPa for 20 min.

3.4 Preparation of sand medium for *in vitro* culture

Washed river sand was obtained from Rockhampton Mini loads and was sieved through to a 2 mm mesh sieve and dried in an oven for two days at 60ºC. Dried sand (29.5 g) was added to each of large test tubes (150 mm x 70 mm) and 20 ml of MS

solution was poured. The test tubes were autoclaved at 121ºC and 103 kPa for 20 min.

3.5 Preparation of MS media with cadmium

Firstly 100 mM of cadmium sulphate solution was prepared by dissolving 25.6 g of $3(CdSO_4)\ 8H_2O$ (analytical grade; Sigma-Aldrich Pty. Ltd) salt in 1000 ml distilled water. MS media was prepared as described in Section 3.1; but the exception was $3(CdSO_4)8H_2O$ stock solution was added to the media before making up the volume to 1000 ml in the conical flask. No cadmium sulphate solution was added to the control, 0.1 ml was added for 10 µM, 0.3 ml for 30 µM, 1 ml for 100 µM, 2 ml for 200 µM, 5 ml for 500 µM and 10 ml for 1000 µM cadmium per litre of the media. The solution was mixed thoroughly using a magnetic stirrer and the pH was adjusted to 5.77. Once a clear solution was obtained, the solution was mechanically dispensed medium sized (105 mm x 44 mm) tissue culture tubes. The tubes were autoclaved at 121°C and 103 kPa for 21 minutes. When the autoclaving was completed, the autoclave's door was gently opened and the tubes were allowed to cool in the autoclave for about 20 minutes. After autoclaving the media pH rose to 6.0 in control, 10 µM and 30 µM but the rest of media (100, 200, 500 and 1000 µM) had lower pH (5.13, 5.38, 5.26 and 5.25) respectively. The tubes were removed and the lids tightened. The tubes were stored at room temperature.

3.6 Preparation of cadmium stock solution

The analytical grade cadmium salt, $3(CdSO_4)\ 8H_2O$ (molecular weight 769.52 g, Sigma-Aldrich Pty. Ltd) contains 3 moles of Cd. Thus one mole of Cd can be obtained from 256 g of the above salt. The stock solution of 100 mM can therefore be obtained by dissolving 25.6 g of the above salt in 1000 ml of distilled water.

3.7 Preparation of hydroponics solution with cadmium

Manutec hydroponics (Manutec Garden Care Products) nutrient salts were purchased from Bunning's Warehouse. Solution was prepared according to manufacturer's instructions 72 grams of Part 1 that contained (N, P, K; 7.6/3.1/18.2 + Trace Elements) was dissolved in a plastic container holding 60 litres of demineralised water. Then 48 g of the Part 2 (Ca (NO_3)2) was added and the pH adjusted to 6.3. The nutrient solution was then dispensed into small containers and the control bucket was left as is and the Cd treated buckets were added with 100 mM of Cd SO_4 stock solution at (per litre) 0.3 m/l for 30 µM, 1 ml for 100 µM, 2 ml for 200 µM, 5 m for 500 µM and 10 ml for 1000 µM cadmium. The pH of the control hydroponics solution was adjusted to 6.3 and the pH of Cd treated solutions was adjusted to 5.3.

3.8 Experimental design and statistical analysis

The experiments were laid out according to completely randomised design (CRD; tissue culture experiments) or randomised complete block design (RCBD; glasshouse experiments) with 3-5 replications.

Analysis of variance was performed using Genstat version 13 following testing the data for normality, outliers and homogeneity of error variances. Standard errors of means were used when the ANOVA was not significant.

3.9 Atomic Absorption Spectroscopy (AAS)

Preparation of standard solutions
Recommended standard materials:
Cadmium metal strip or granules 99.99%
Cadmium sulphate A.R. Grade $3CdSO_4.8H_2O$ (molecular weight 769.52 g)

Dissolve 1.000 g of cadmium sulphate in a minimum volume of 1:1 nitric acid.

Dilute to 1 litre to give 1000 µg/L Cd.

Recommended instrument parameters

Atomic absorption

Working conditions (Fixed)	
Lamp current (Note 1)	4 mA
Fuel (Note 2)	acetylene
Support	air
Flame stoichiometry	oxidizing

Note 1: Absorbance depends on lamp current

Note 2: Absorbance sis dependent on flame stoichiometry. Adjust the fuel flow carefully for maximum sensitivity.

Working conditions (Variable)

Wavelength	Slit width	Optimum working range
nm	nm	µg/ml
228.8	0.5	0.02-3
326.1	0.5	20-1000

Flame emission

Wavelength	326.1 nm
Slit width	0.1 nm
Fuel	acetylene
Support	nitrous oxide

Note: cadmium is not usually determined by flame emission because of the poor emission characteristics of the element. However due to limitations in the availability of the equipment, flame emission AAS was used and the sample Cd concentrations were estimated based on standard curve.

Chapter 4

Optimising growth conditions for establishing tomato plants in tissue culture

4.1 Summary

Tomato is one of the most important vegetable crops both for commercial use and for its utilisation in scientific studies such as genetic engineering and genetic transformation. The first experiment was conducted to test seed germination and to optimise shoot multiplication procedures from hypocotyls, cotyledons and shoot tips of 15 genotypes of tomato using Murashige and Skoog (MS) basal medium. The second experiment was conducted in tissue culture using three genotypes of tomato. Seed germination percentage, shoot height, fresh shoot weight and dry shoot weight were determined for plants grown in MS media and sown with 3, 6, 9 and 12 seeds using five replications. The third experiment was conducted using three genotypes of tomato treated with five different media (agar, wiped slant agar, unwiped slant agar, potting and sand media) for 5 weeks.

4.2 Introduction

Tomato is an important vegetable crop which is grown all over the world. It belongs to the family Solanaceae. *In vitro* technique is a useful method for plant multiplication and genetic studies (Lai and Liu 1982). This chapter describes a protocol for regeneration of plants from shoot tip, hypocotyl and cotyledon. The type of explants and the genotypes used, the type of culture media and growth hormones included, quality of agar and sucrose, light intensity and temperature affect plant

regeneration in tissue culture (Jabeen *et al*. 2005). Hypocotyl, cotyledon, pedicel and peduncle are the main explants used for callus formation and multiplication (Mohamed *et al.* 2010). Proliferation of genetically exact duplicates and genetic metamorphosis procedures can be applied via micropropagation, organogenesis or somatic embryogenesis (Gubis *et al*. 2004).

The aim of this experiment was to test responses of 15 genotypes of tomato and different parts of the plants (hypocotyls, shoot tips and cotyledons) *in vitro* conditions.

The main objectives are:

1. To optimise tissue culture procedures for establishing plants *in vitro.*
2. To determine plant growth conditions for establishing plants in tissue culture.
3. Assess relative response of different explants: hypocotyl, shoot tips and cotyledons to tissue culture conditions

4.3 Materials and Methods

4.3.1 Collection of tomato seeds

Seeds of fifteen genotypes of tomato, viz., Rouge de Marmanda, Red Cherry, San Marzano-2, Grosse Lisse, Heir Loom, Mortgage Lifter, Roma VF, Oxheart, Tiny Tom, Apollo Improved (produced by Mr. Fothergill's seeds Pty. Ltd. Homebush, NSW, Australia), Burke's Backyard (produced by Yates Italian tomato seeds, NSW, Australia) and Big Beef (produced by Yates Padstow, NSW, Australia) Roma (produced by Grower's Pride, Hortico Pty. Ltd, NSW, Australia) were purchased from Bunning's Warehouse and IGA super market, and cultivar Nash 1 and Nash 2 were kindly provided by Dr Nanjappa Ashwath, Central Queensland University.

4.3.2 Procedure for preparing agar medium

The Murashige and Skoog's (1962) tissue culture media was prepared as described in Chapter 3.1. The MS solution was mechanically dispensed into 10 ml tubes using automatic dispenser. All test tubes were placed in the autoclave and sterilised at 121ºC and 103 kPa for 21 minutes. Seeds of 15 genotypes of tomato were surface sterilised with 25% commercial bleach (containing 1% sodium hypochlorite) for 5 min. The seeds were rinsed 3 times with sterile water. Three tomato seeds were placed in each test tube and the experiment was replicated 5 times. The pH of the solution was adjusted to 5.7. All tissue culture tubes were placed in a controlled environment room and the temperature was maintained at 25˚C. Tissue culture tubes were exposed to light (ca 50 m^2 s^{-1}) provided by cool white fluorescent light for 16 hours.

4.3.3 Selecting responsive plant parts

Seeds were germinated in MS media for one week. The one week-old seedling was separated into hypocotyls, cotyledons and shoot tips in a laminar flow cabinet. These parts were then aseptically transferred to new MS medium. The tissue culture tubes containing explants were incubated in a controlled environment room. The temperature of the controlled environment room was maintained at 25˚C at all times and the tubes were exposed to 16 hour light provided by cool white fluorescent tubes.

4.3.4 Shoot regeneration from hypocotyls, shoot tips and cotyledons

The number of shoots produced by each of the explants (hypocotyls, shoot tips and cotyledons) was counted after 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 weeks of inoculation.

4.3.5 Callus formation by different explants

Explants were observed for callus induction and the number of protrusions produced by each of the explants was counted and used in the analysis.

4.3.6 Statistical analysis of data

Analysis of variance was performed using Genstat version 13 following testing: the data for outliers and homogeneity of error variances. Standard errors of means or least significant differences (lsd) were used for comparing the means.

4.4 Results

4.4.1 Seed germination

The lowest seed germination (67%) was observed in Heir Loom (Fig. 4.1) followed by Tiny Tom. The highest seed germination (100%) was found in the genotypes Red Cherry, Mortgage lifter, Burke's Backyard, RomaVF, Oxheart, Roma and Apollo Improved. There were significant (P<0.004) differences between genotypes in seed germination (Table 4.1).

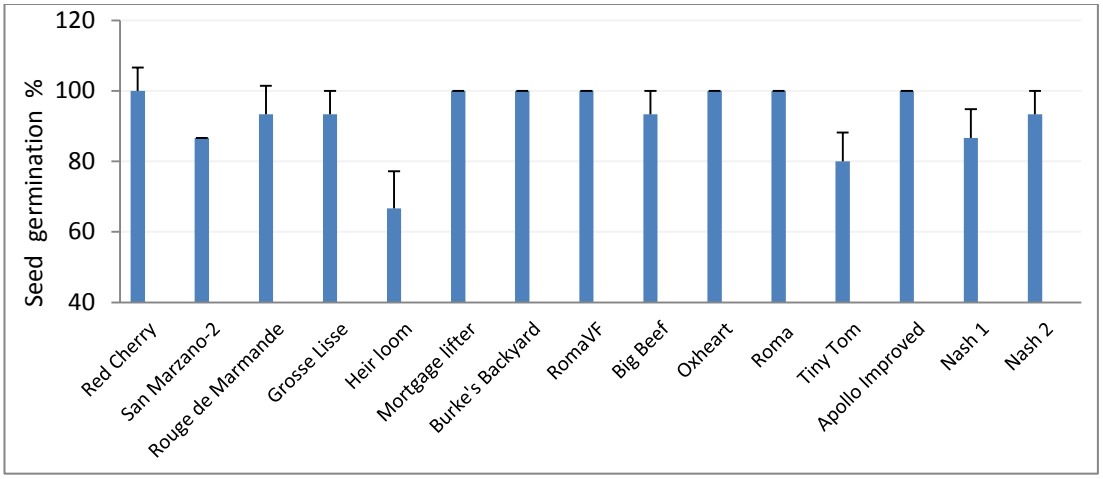


Figure 4.1 Seed germination percentage in 15 genotypes.
Bars represent SE (n=3).

Table 4.1 *P* values from ANOVA for 15 genotypes of tomato

	Df	Ger%	Cot Call	Cot shoot	Hyp Call	Hyp	StipCall	Stip
Cultivar	14	0.004**	<.001***	0.002**	0.878 ns	0.097 ns	0.148 ns	0.721 ns

Df (n-1)=degree of freedom, Ger%=germination percentage, Cot shoot=number of shoots from cotyledons, Cot Callus=callus formation from cotyledon, Hypo=number of shoots from hypocotyls, Hypo Callus=number of callus formation from hypocotyl, S tip=number of shoot tips, StipCall=number of callus from shoot tip.

4.4.2 Shoot regeneration from the explants

Amongst the three explants used, hypocotyls responded better than the shoot tips and cotyledons for shoot regeneration. The highest number of shoots (14.6) was obtained from hypocotyls of Rouge de Marmande (Fig. 4.2) and the lowest from Heir Loom. Root formation was noticed in most of the cotyledons in all genotypes. The hypocotyls also produced roots but the roots were not so prolific unlike in cotyledons. The shoot tips rarely produced roots. These results suggested that cotyledons are the best explants for the production of rooted seedlings from tomato.

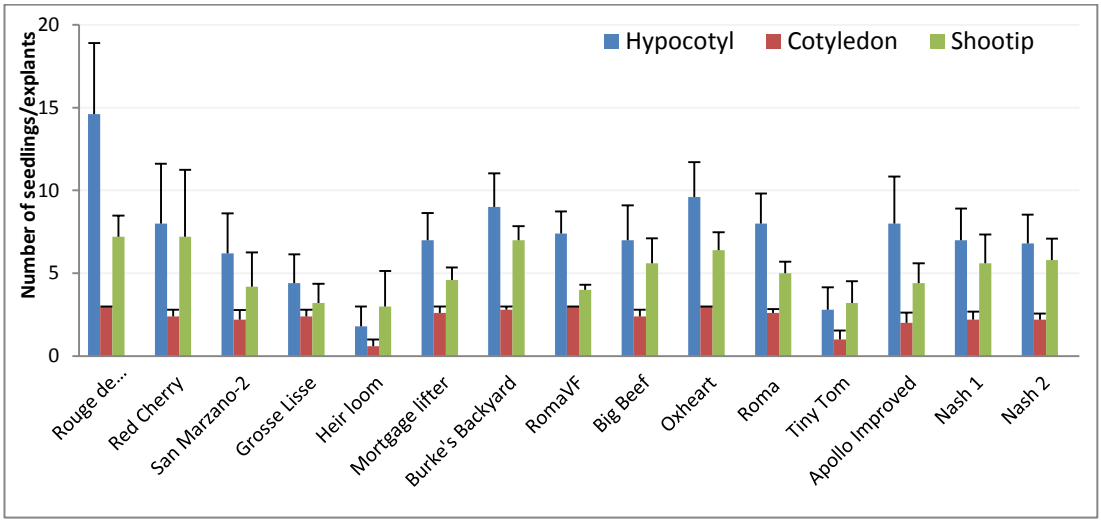


Figure 4.2 Number of seedlings produced by hypocotyls, cotyledons and shoot tips of 15 genotypes. Bars represent SE (n=3).

4.4.3 Callus formation

The numbers of calli protrusions formed by the explants were counted and cotyledons of some genotypes developed more calli than hypocotyls or shoot tips (Fig. 4.3). Overall, the genotypes produced 1-2 calli protrusions per explant. Nine of the 15 genotypes developed no callus from their hypocotyls.

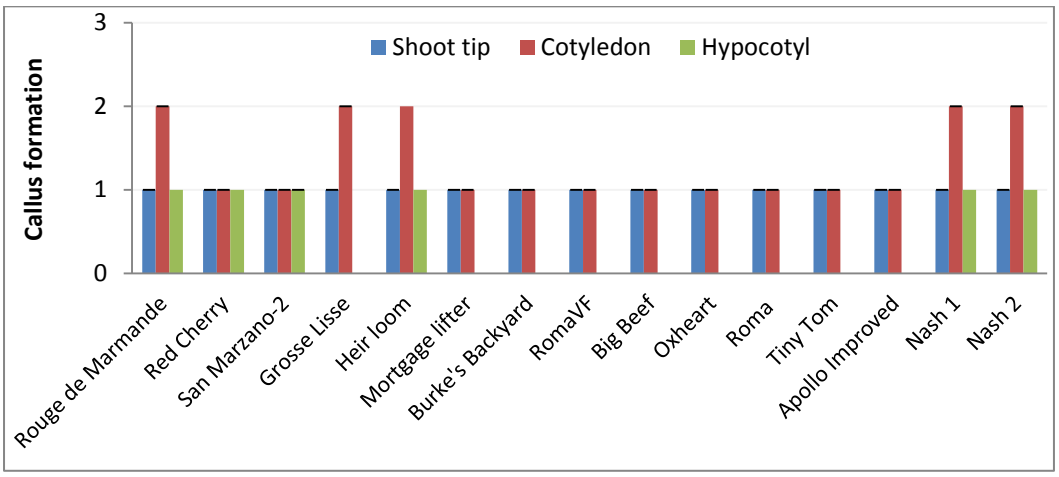


Figure 4.3 Callus formations from shoot tips, cotyledons and hypocotyls of 15 genotypes of tomato.

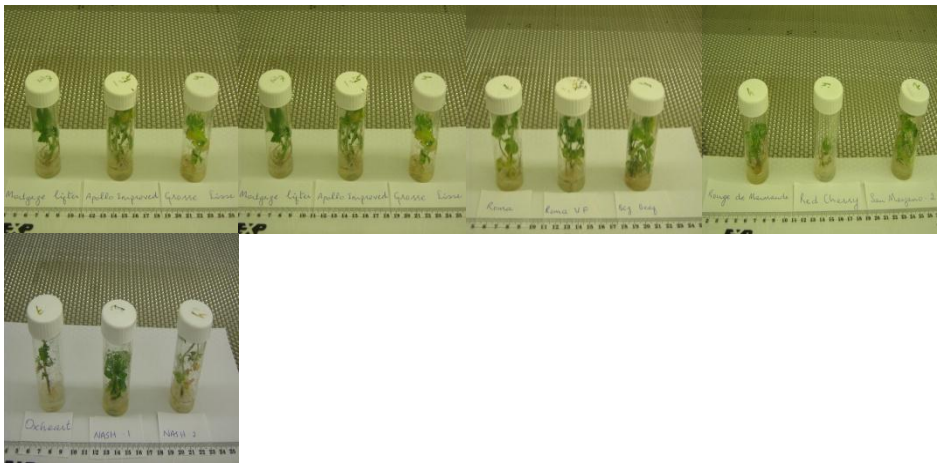


Plate 4.1 Photos of seedlings raised in tissue culture 5 weeks after sowing. From left to right (Mortgage lifter, Apollo Improved, Grosse lisse, Burke's Backyard, Tiny Tom, Hire loom, Roma, RomaVF, Big Beef, Rouge de Marmande, Red Cherry, San Marzano-2, Oxheart, Nash1 and Nash2)

4.5 Discussion

In this experiment, 14 genotypes showed more than 80% seed germination. The lowest germination was found in Heir loom (66.7%). The highest seed germination

(100%) was found in 7 of 15 genotypes. Ganesan *et al.* (2007) reported 85% germination in Okra (*Abelmoschus esculentus*) grown in MS basal medium.

In this study, the numbers of seedling produced from hypocotyls were higher than those from cotyledons and shoot tips in all genotypes except in the genotype Heir Loom and Tiny Tom. Fewer seedlings were obtained from cotyledon than from hypocotyls and shoot tips in all genotypes except in Grosse Lisse, Heir Loom and Tiny Tom. Jabeen *et al*. (2005) reported that the higher numbers of seedling were produced from hypocotyls and shoot tips in tomato cultivar Riograndea grown in MS medium and growth regulators (zeatin and indole-3-acetic acid) compared to tomato cultivar Roma, Money maker, Nagina and Feston. Results from the current study are consistent with those reported by Jabeen *et al.* (2005).

In the tomato cultivar Premium and Hana, the number of seedlings from hypocotyls was higher than those produced from the cv. Money maker (Gubis *et al*. 2004). In tomato cultivar Pearl and Beril, no callus formation was found in cotyledons supplemented with MS basal medium, but the production of shoots and callus was observed in the hypocotyls of tomato cultivar Pearl and Beril (Mohamed *et al*. 2010). The current results of producing higher number of calli in hypocotyls than in cotyledons contradict the observations of Mohamed *et al*. (2010).

In vitro culture MS medium supplemented with 1.0 mg/l benzyladenine produced the same number of seedlings from shoot tips in tomato cultivar Omdurman when compared to control (Ishag *et al*. 2009). The best response of callus induction was found in Okra cultivar Surabhi supplemented with MS salt, growth regulators and sucrose when compared to others such as glucose, fructose and maltose (Ganesan *et al.* 2007). Callus formation and seedlings from shoot tips increased in Turkish

cowpea cultivar Akkiz in MS basal medium supplemented with 0.5 mg/l benzylaminopurine (Aasim *et al*. 2008). The higher shoot organogenesis was observed in tomato cultivar Ailsa Craig, UC82B and Rutgers in MS medium without growth regulators compared to Bell pepper cv. Piquillo, Yolo Wonder, Permagreen and Golden summer (Pozueta-Romero *et al*. 2001). The number of plants producing callus from shoot tips increased in Indian cultivar *Withania somnifera* in MS basal medium supplemented with naphathalene acetic acid (0.5 µM); (Silva and Senarath 2009).

In conclusion, 100% seed germination was found in 7 of the 15 genotypes of tomato. The seedlings obtained from hypocotyls were potential explants for *in vitro* shoot multiplication of fifteen genotypes of tomato. Higher callus formation from cotyledons was observed in 5 genotypes. Plant tissue culture technology helps to propagate plants rapidly and in an efficient manner.

4.6 Optimising the number of seeds to be used in tissue culture

Plant regeneration and embryo induction in *in vitro* cultures are dominated by the type of genes and physiological status of explants (Sheeja *et al.* 2004). Tomato contains useful genes that can be exploited in horticulture and it is also useful for genetic engineering (Jabeen *et al.* 2005). Tomato could be grown and regenerated using *in vitro* culture techniques. Seed raised plants are robust and are tolerant to virus diseases (Sabongari and Aliero 2004).

The aim of this experiment was to optimise the number of plants to be grown in each tissue culture tube to maximise biomass production using response of three tomato genotypes, Burke’s Backyard, Red Cherry and San Marzano-2.

The major objectives are:

1. Determine the number of seeds to be sown per tube
2. Assess health and vigour of the plants grown at different seed densities
3. Compare growth rates of the three genotypes used in tissue culture

4.7 Materials and Methods

4.7.1 Selection of tomato genotypes

Three genotypes of tomato, viz. Burke's Backyard, Red Cherry and San Marzano-2 (produced by Mr. Fothergill's Seeds Pty Ltd. South Windsor, NSW and Australia) were selected from previous experiments and the seeds of these genotypes were purchased from Bunning's Warehouse.

4.8 Preparation of agar medium

Tissue culture (MS media) was prepared as described in Chapter 3.1. The solution (25 ml) was mechanically dispensed into each medium tube using media dispenser. All test tubes were autoclaved at 121°C and 103 kPa for 21 min. The pH of the solution was adjusted to 5.7 before autoclaving. Seeds of the three genotypes were surface sterilised with 25% commercial bleach (which contained 1% sodium hypochlorite) for 5 min. The seeds were rinsed 3 times with sterile water. Then 3, 6, 9 and 12 tomato seeds were placed on each tube and incubated in a controlled environment room using five replications. The temperature was maintained at 25°C and cool white fluorescent light was provided light for 16 hours.

4.9 Plant growth parameters

The seed germination and shoot height were monitored at 7, 14, 21 and 33 days after inoculation. Leaf and stem of each plant was harvested after 33 days of inoculation

and were rinsed thoroughly with distilled water and weighed to determine fresh weight. Separated leaves and stems were placed in a dryer at 72°C for 3 days and weighed again to determine dry weight.

4.10 Statistical analysis of data

Analysis of variance was performed using Genstat version 13 following testing: the data for outliers and homogeneity of error variances. Standard errors of means or least significant differences (lsd) were used for comparing the means.

4.11 Results

4.11.1 Seed germination

A hundred percent seed germination was recorded in Burke's Backyard when 6 and 12 seeds per tube were used (Fig. 4.4). Seed germination was not affected by the the number of seeds used in Burke's Backyard, San marzano-2 and Red Cherry.

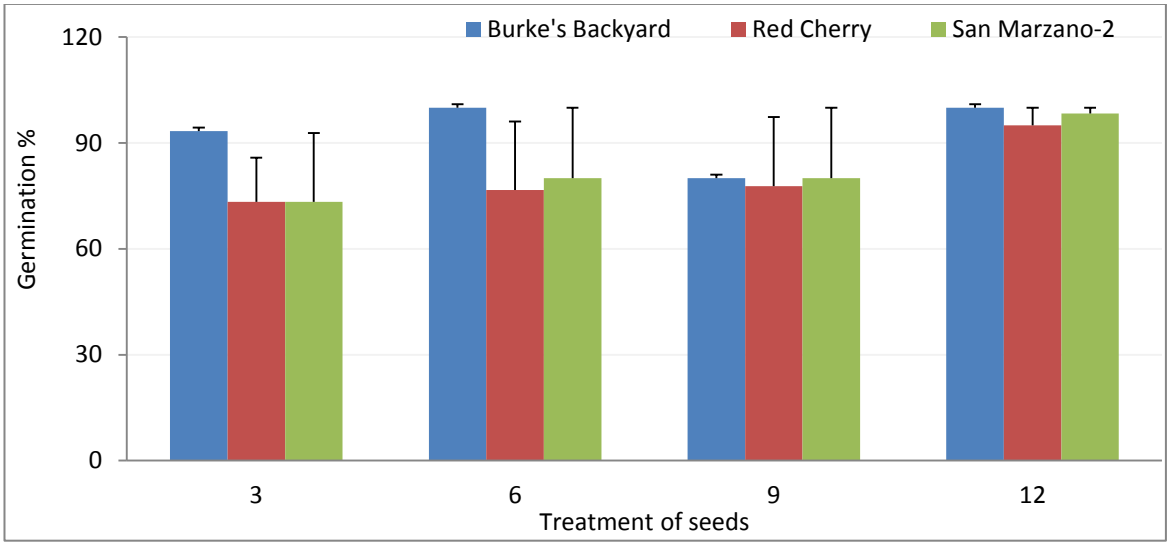


Figure 4.4 Seed germination % in 3 genotypes, Burke's Backyard, Red Cherry and San Marzano-2that were treated with 3, 6, 9, 12 seeds.
Bars represent SE (n=3).

4.11.2 Shoot height

Shoot height was little affected by the number of seeds used (Fig. 4.5). The tallest seedlings were found in Red Cherry where 3 or 6 seeds per tube were used compared to Burke's Backyard and San Marzano-2. The shoot height of Burke's Backyard was shorter than that of Red Cherry. Shoot height was not different in all genotypes treated with 9 seeds.

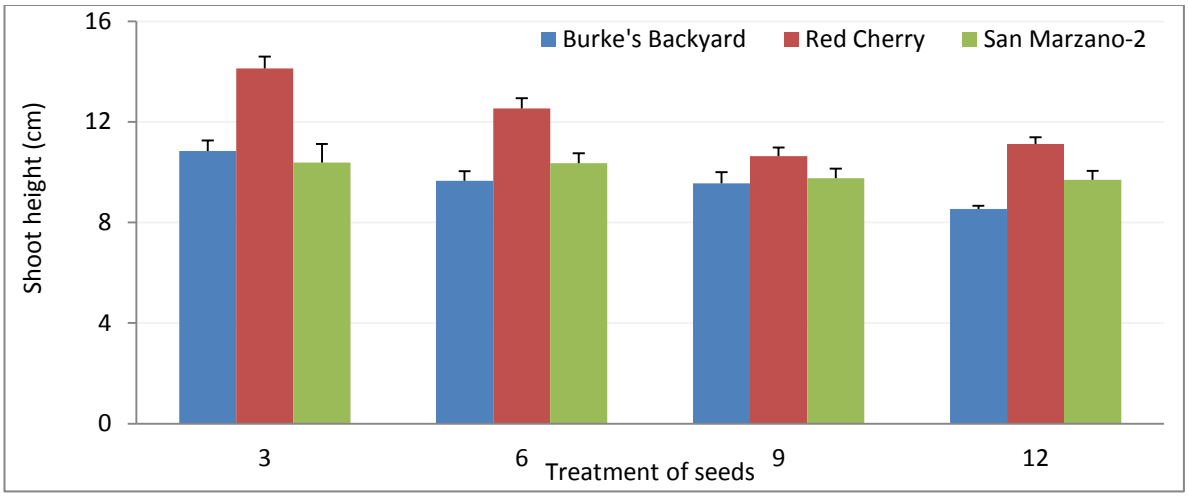
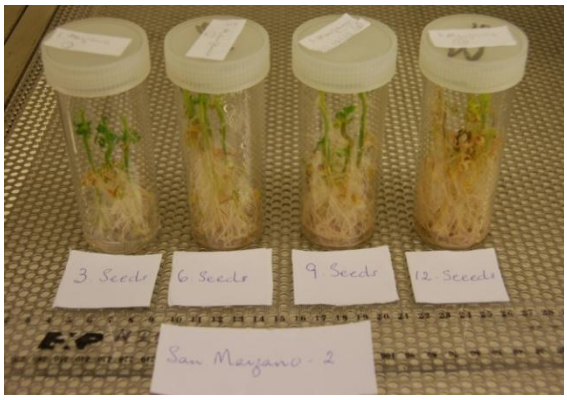


Figure 4.5 Plant shoot height (cm) in 3 genotypes after 5 weeks.
Bars represent SE (n=3).

(A)

(B)



(C)

Plate 4.2 Genotype Red Cherry (A), Burke's Backyard (B) and San Marzano-2 (C) with four treatments 3, 6, 9 and 12 seeds per tube.

4.11.3 Leaf fresh weight

The leaf fresh weight increased with an increase in the number of plants per tube (Fig. 4.6). Amongst the three genotypes, San Marzano-2 treated with 6, 9 and 12 seeds per tube had higher weights than those with 3 seeds per tube after 5 weeks. The lowest fresh weight was recorded in all three genotypes treated with 3 seeds. The leaf fresh weight was significantly (P<0.05) different amongst the three genotypes that had 9 seeds per tube.

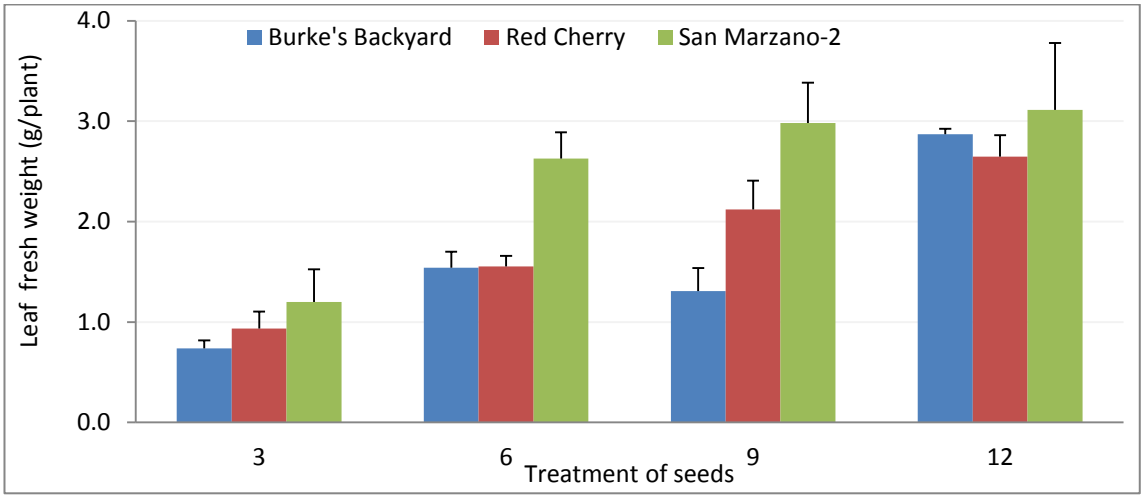


Figure 4.6 Leaf fresh weights (g/plant) of 3 genotypes after 5 weeks.
Bars represent SE (n=3).

4.11.4 Leaf dry weight

The leaf dry weight was highest in treatments receiving 12 seeds per tube. Treatments with 6 or 9 seeds per tube had similar dry weight but those with three seeds had the lowest dry weight (Fig. 4.7).

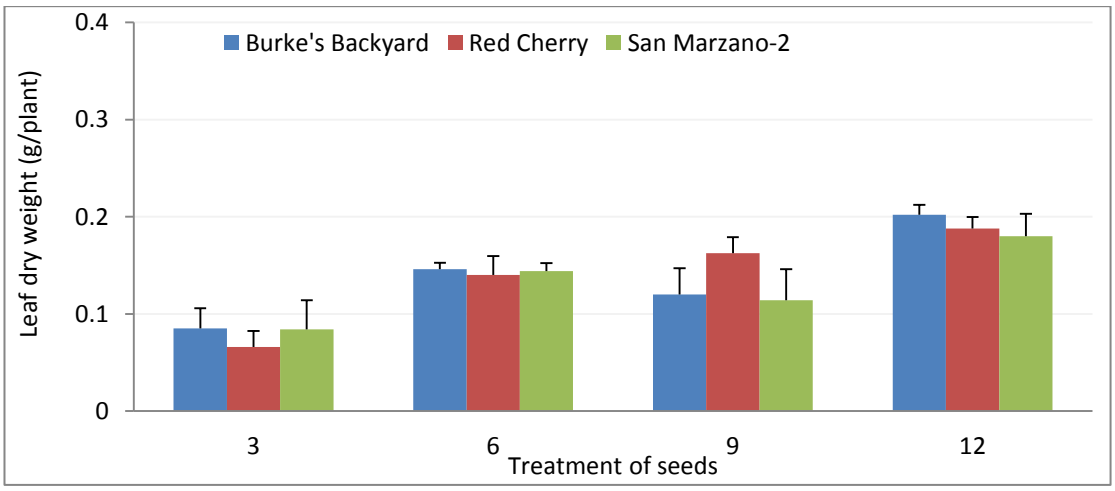


Figure 4.7 Leaf dry weights (g/plant) of three genotypes after 5 weeks.
Bars represent SE (n=3).

Table 4.2 *P* values from ANOVA for 3 genotypes

	Df	G%	Ht	FW	DW
Cultivar	2	0.059*	0.003*	0.010*	0.476 NS
Treatment	3	0.010**	0.03*	<.001***	<.001***
Cultivary*treatment	6	0.546 NS	0.4 NS	0.307 NS	0.042*

Df (n-1)=degree of freedom, G%=germination percentage, Ht=plant height, FW=fresh weight, DW=dry weight

4.12 Discussion

In the present experiment, 100% seed germination was observed in Burke's Backyard sown with 6 and 12 seeds *in vitro*. Above 73% seed germination was observed in all three genotypes treated with 3, 6, 9 and 12 seeds. Sathyanarayana *et al*. (2008) reported 100% seed germination in legume *Mucuna pruriens* var. *utilis* grown in MS basal medium, growth regulators and without sucrose. Ninety six per cent germination was observed in tomato *Lycopersicon esculentum* var. Omdurman supplemented with full strength MS medium, and 100% seed germination was found in this same cultivar supplemented with half-salt MS medium (Ishag *et al*. 2009).

In the present investigation, the Red Cherry produced taller seedlings than Burke's Backyard and San Marzano-2 in treatments receiving 3, 6 and 12 seeds. The shoot length decreased in the legume *Mucuna pruriens* var. *utilis* in MS basal medium supplemented with 6-benzylamino purine (13.32 µM) compared to the media containing no growth regulator (Sathyanarayana *et al*. 2008). The shoot length increased in citrus cultivar Kinnow and Feutrell's treated with MS salt (Murashige & Skoog 1962) compared to MT salt (Murashige and Tucker 1969), (Fatima *et al.* 2010). The shoot length increased in *Dendrocalamus as per* supplemented with MS medium and without plant growth regulator when compared to MS medium supplemented with benzyladenine 7 mg/l (Arya *et al*. 1999).

In the present study, higher fresh weight production was observed in Burke's Backyard, Red Cherry and San Marzano-2 treated with 12 seeds compared to those sown with 3 seeds. Higher fresh weight was also found in the tomato cultivar Hana Queen in MS medium (Kubota *et al*. 2001). The shoot fresh weight increased in

cultivar *Targets minute* supplemented with half-strength MS medium and indole-3-acetic acid (IAA) compared to the same cultivar supplemented with ½ strength MS, IAA and 60,000 µM mannitol (Mohamed, 2000).

In the present work, the dry weight increased in all three genotypes treated with 12 seeds compared to 3 seeds. Higher dry weight was found in the tomato cultivar Hana Queen in MS medium and photoautotrophic micropropagation compared to conventional photomixotrophic micropropagation without leaves (Kubota *et al*. 2001). Shoot dry weight increased in cultivar *Tagetes minuta* supplemented with half strength MS medium and indole-3-acetic acid (IAA) compared to the same cultivar supplemented with half strength MS, IAA and 60,000 µM mannitol (Mohamed, 2000).

In conclusion, the growth of three tomato genotypes Burke's Backyard, Red Cherry and San marzano-2 were investigated using 3, 6, 9 and 12 seeds per tube. The higher seed germination was observed in all three genotypes treated with12 seeds per tube. The genotype Red Cherry produced taller seedlings than Burke's Backyard and San Marzano-2 when they were treated with 3, 6, 9 and 12 seeds. The high biomass was found in all three genotypes treated with 12 seeds of tomato. The genotype Red Cherry and San Marzano-2 had better growth than Burke's Backyard when 12 seeds per tube were used.

4.13 Selection of suitable media for growing tomato plants in *in vitro* culture

Tissue culture technique is an essential tool for regeneration of endangered plant species. Micropropagation technique is a fundamental step in transformation and genetic engineering processes and provides disease free plantlets. Micropropagation

techniques are useful for the production of woody plants for reforestation (Bell *et al*. 1993). Selection of explants, culture media and bacteria environment is important for regeneration of plants *in vitro* (Mingozzi *et al*. 2008). Atmospheric conditions, pollination and before and after seed spread, and seed granivory may affect seed germination and production (Burgos *et al*. 2007). Seed germination percentage, and growth rate of seedling is determined by genotypes, temperature and relative humidity (Islam *et al*. 2009). Tough seeds that are drowned in water over night before sowing showed the lowest mortality and high seed germination. Seeds are treated before sowing to help them absorb water leading to higher seed germination (Islam *et al.* 2009).

The aim of this experiment was to compare the effects of five plant growth media (agar, wiped slant agar, unwiped slant agar, potting media and sand media) on the growth of three genotypes of tomato (San Marzano-2, Red Cherry and Oxheart).

The specific objectives include:

1. Select best media amongst agar, wiped slant agar and unwiped slant agar media for screening tomato seedlings *in vitro*
2. Test if potting mix and sand culture media are appropriate for using them in *in vitro* culture of tomato plants.

4.14 Materials and Methods

4.14.1 Cultivar selection

Three genotypes viz., San Marzano-2, Red Cherry and Oxheart (produced by Mr.Fothergill's Seeds Pty Ltd. South Windson, NSW and Australia) were selected

from previous experiment, and the seeds of these genotypes were purchased from Bunning's Warehouse.

4.14.2 Media preparation

Forty five large plastic test tubes (150 mm x 70 mm) were collected and arranged in tissue culture laboratory. Agar medium was prepared in tissue culture laboratory. Potting mix (Yates Premium Potting mix New Zealand Limited, Onehunga, Auckland, New Zealand) was purchased from Bunning's Warehouse and sand was collected from Rockhampton Mini Loads.

The procedures used in preparing the media, potting mix and the sand media are described in Chapter 3.

4.14.3 Plant growth parameters

Seed germination and plant shoot height were monitored at 7, 14, 21 and 33 days after inoculation. The seedlings from each tube were harvested and rinsed thoroughly with distilled water and separated into leaves, stems and roots. The tissues were blotted dry with paper towels and then placed in a dryer at 72˚C for 3 days before determining dry weight.

4.14.4 Statistical analysis of data

Analysis of variance was performed using Genstat version 13 following testing: the data for outliers and homogeneity of error variances. Standard errors of means or least significant differences (lsd) were used for comparing the means.

4.15 Results

4.15.1 Seed germination

In the tissue culture experiment treated with agar media, wiped slant agar and sand media, 100% seed germination was found in Oxheart. In genotype Red Cherry 100% seed germination was found in unwiped slant agar media (Fig. 4.8). Eighty nine percent seed germination was observed in San Marzano-2 and Red Cherry in wiped slant agar media and sand media. Genotypes San Marzano-2 and Oxheart also had 89% of seed germination in treatments unwiped slant agar media and potting media. The low seed germination (78%) was observed in San Marzano-2 and Red Cherry in agar media and Red Cherry in potting media. No significant differences were found in seed germination between the three genotypes and the media treatments.

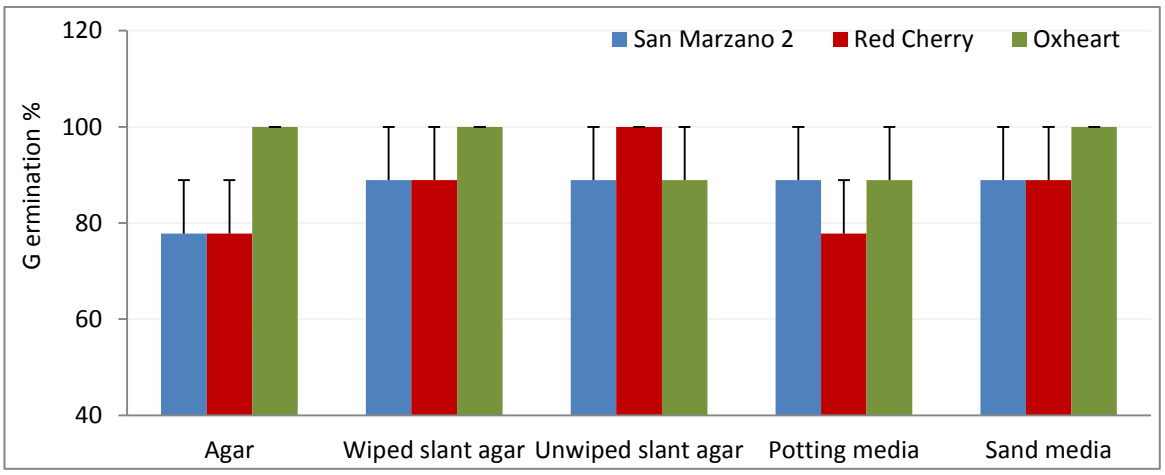


Figure 4.8 Seed germination % of 3 genotypes San Marzano-2, Red Cherry and Oxheart supplemented with 5 different media.
Bars represent SE (n=3).

4.15.2 Shoot height

Shoot height was lowest in agar media than in other media. The wiped slant agar seems to produce the best conditions for seedling growth. Sand and potting media had similar effect as the agar media.

Amongst the three genotypes, San Marzano-2 grew best in slant agar, where as the Red Cherry grew equally well in all except the agar media. The growth of San Marzano-2 was significantly reduced in potting media and sand media. (Fig. 4.9; Plate 4.3)

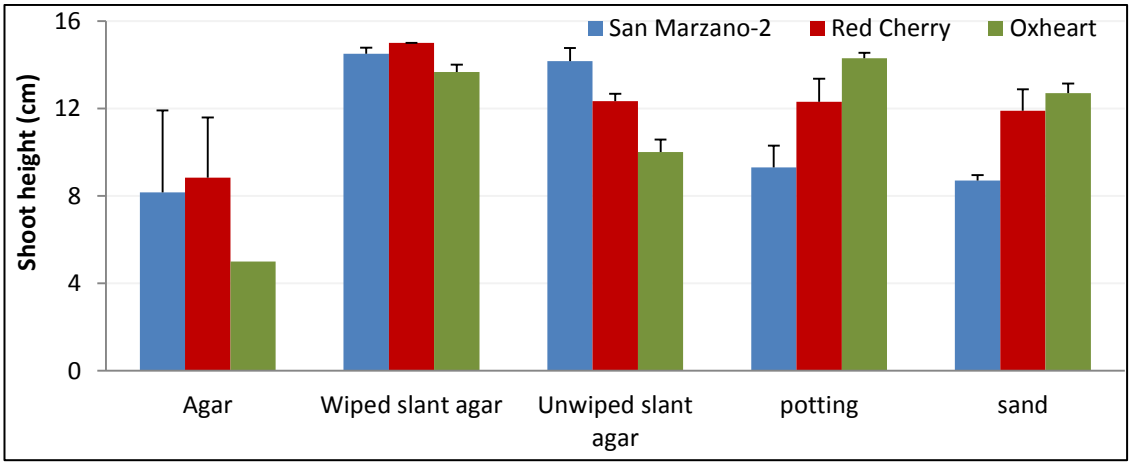


Figure 4.9 Shoot heights (cm) of 3 genotypes after 5 weeks.
Bars represent SE (n=3).

Plate 4.3 Shoot growth of San Marzano-2 in five different media left to right sand, potting mix, agar, unwiped slant agar and wiped slant agar media

4.15.3 Shoot dry weight

The highest shoot dry weight was found in Red Cherry (0.64 g) treated with agar medium compared to the other media and genotypes (Fig. 4.10). The shoot dry weight of San Marzano-2 and Red Cherry were higher than those of Oxheart treated

with agar media. The shoot dry weight of San Marzano-2 decreased in wiped slant agar media compared to Oxheart and Red Cherry. The shoot dry weight of San marzano-2 (0.5 g) was higher than Oxheart (0.15 g) and Red Cherry (0.2 g) in unwiped slant agar media. In the potting media shoot dry weight of Oxheart performed better than other genotypes.

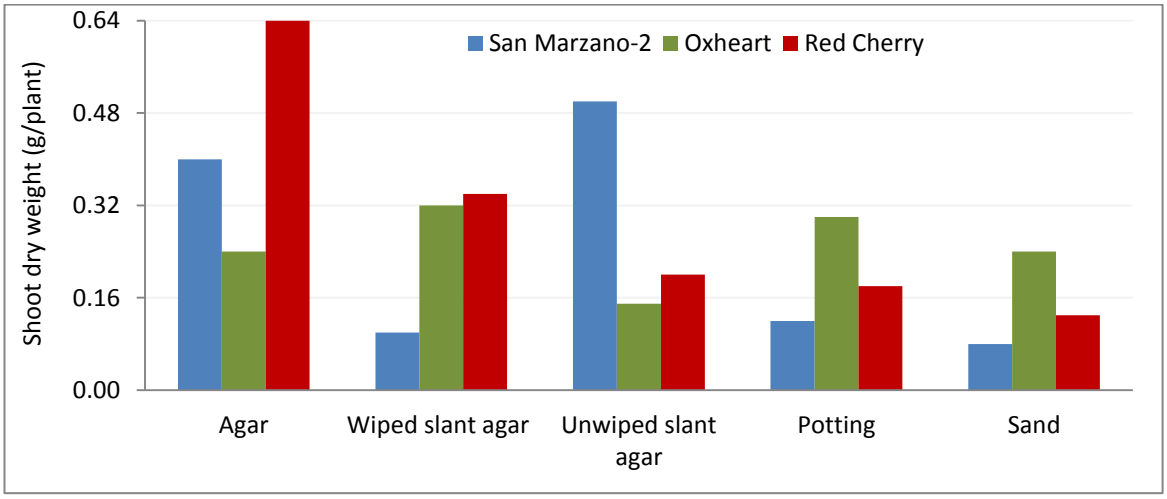


Figure 4.10 Shoot dry weights of 3 genotypes after 5 weeks.

4.15.4 Root dry weight of three genotypes in five growth media

The root dry weight was high in San Marzano-2, Oxheart and Red Cherry in agar media as compared to others media (Fig. 4.11). The root dry weight was high in San Marzano-2 in sand media as compared to wiped slant agar media and potting media. The lower root dry weight was found in Red Cherry in unwiped slant agar media and sand media as compared to agar media.

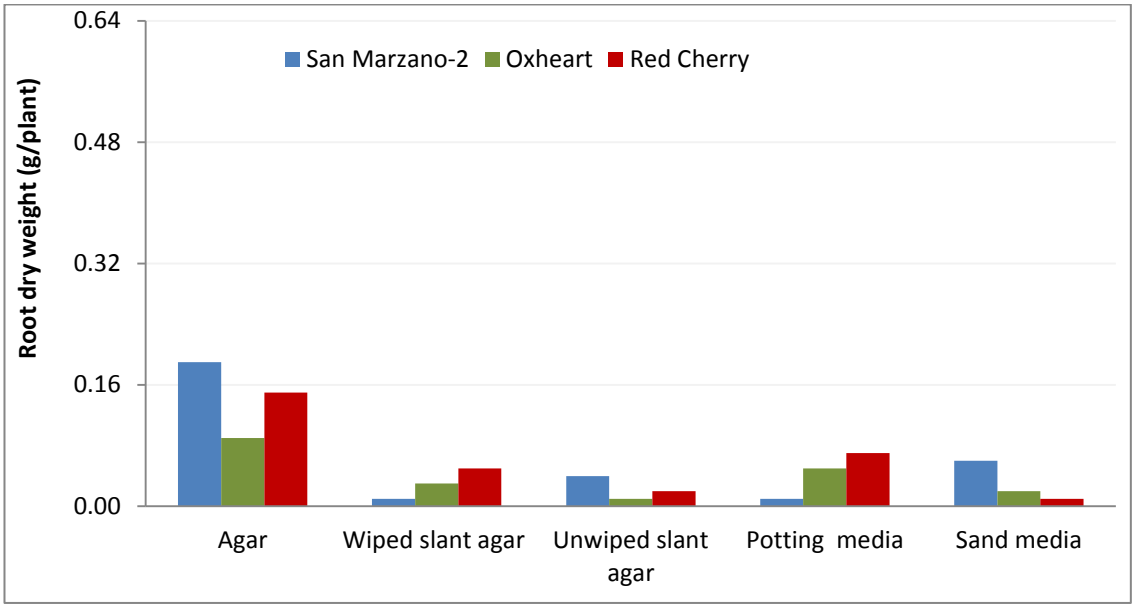


Figure 4.11 Root dry weights of 3 genotypes after 5 weeks

Table 4.3 *P* values from ANOVA for shoot dry weights (g/plant) for 3 genotypes

	Df	G%	Ht	FW	DW
Cultivar	2	0.059*	0.003*	0.010*	0.476 NS
Treatment	3	0.010**	0.03*	<.001***	<.001***
Cultivarv*treatment	6	0.546 ns	0.4	0.307 ns	0.042*

Df (n-1)=degree of freedom, G%=germination percentage, Ht=height, FW=fresh weight, DW=dry weight

4.16 Discussion

In the presents study, more than 78% seed germination was observed in all three genotypes grown in five media. A hundred percent seed germination was observed in Oxheart treated with agar, wiped slant agar medium and sand medium. The genotype Red Cherry also showed 100% seed germination in unwiped slant agar media. Abrie and Staden (2001) reported 75% germination in *Aloe polyphylla* at 24˚C. In the cultivar *Gymnema sulvestre*, 62% seed germination was found in *in vitro* (Komalavalli and Rao 2000). Sixty percent of seed germination was observed in tomato (*Lycopersicon esculentum*) treated with solid medium (M11) containing MS full strength basal media and plant growth regulator (Bhattarai *et al*. 2009).

Chaudhry *et al*. (2010) found 69% germination in tomato cultivar Money Maker in MS medium.

In this experiment, shoot height was not different in all genotypes treated with agar and wiped slant agar media. There was a significant difference between 3 genotypes in unwiped slant agar, potting and sand media. Bhattarai *et al*. (2009) reported highest shoot height in tomato cultivar CLN1621L, 60 days after pollination. The shoot length increased in cultivar *Gymnema sulvestre* treated with MS media when compared to other media Gamborg (B5), and woody plant medium (WPM) (Komalavalli and Rao 2000).

In this study, the highest shoot dry weight was observed in Red Cherry treated with agar media. Higher shoot weight was found in San Marzano-2 treated with unwiped slant agar when compared to other four media. The shoot weight decreased in San Marzano-2 in wiped slant agar media and potting and sand media when compared to agar media. The shoot dry weight increased in tomato cultivar Bonny Best inoculated with *in vitro* bacterisation compared to the nonbacterised control (Sharma and Nowak 1998). The highest shoot dry weight was observed in tomato cultivar mutant plant MTG where as the lowest shoot dry weight was found in cultivar TG-5 *in vitro* (Kulkarni and Deshpande 2007). The shoot dry weight increased in tomato cultivar CLN1621L, 50 days after pollination *in vitro* compared to 60 days and 70 days after pollination (Bhattarai *et al*. 2009).

In the present experiment, root dry weight was lower than shoot dry weight. The highest root dry weight was observed in San Marzano-2 in agar media. A very small amount of root dry weight was found in all three genotypes in wiped slant agar, potting media and sand media. The higher root dry weight was observed in all three

genotypes treated with agar media when compared to wipe slant agar, potting and sand media. The highest root dry weight was found in tomato cultivar that was drought resistant Hy-3, but the lowest root dry weight was observed in drought susceptible tomato cultivar TG-5 *in vitro* (Kulkarni and Deshpande 2007). The root dry weight increased in tomato cultivar Bonny Best inoculated with *in vitro* bacterialisation compared to nonbacterialisation (Sharma and Nowak 1998).

In summary, this experiment demonstrates that different genotypes grow best in different media and not all media are suited for all the genotypes being tested. It is also interesting to see the dry matter accumulation in potting media and sand in comparison with the agar media. Clearly, most dry matter was produced from agar media than in potting or sand media. Another interesting feature was that the root biomass was highest in agar media than in other media. Based on this experiment, it was decided to use agar media for screening for cadmium tolerance as the three genotypes grew well in this medium and they also produced high root and shoot biomass.

Chapter 5

Screening of 25 genotypes of tomato for cadmium accumulation in the shoots in *in vitro* culture

5.1 Introduction

Cadmium is a non-essential element for plant nutrition and it inhibits plant growth and development (Grato *et al.* 2008). It is highly mobile in the soil environment (Zhang *et al.* 2009). The cadmium tolerance and accumulation in plants vary between species. The majority of plants are susceptible even to low concentration of cadmium as it causes inhibition of shoot growth, through reduced photosynthesis and nutrient uptake (Rodriguez-Serrano *et al*. 2006). The cadmium toxicity symptoms in plants include discoloration, necrosis, leaf curl and mortality of plant tissues (Zhang *et al.* 2009). In humans it causes unborn baby growth retardation, lack of physical and mental ability and duodenum cancer when they consume food that contains high levels of Cd in their daily diets (Arora, 2008). Cadmium resistant plants show normal functioning in the cells due to glutathione synthesis which leads to reduced Cd toxicity (Wu *et al.* 2004).

The micropropagation method can help produce large number of seedlings for use in remediation of metal polluted sites (Xu *et al*. 2008). Speed of germination and vigour of seedling growth are determined by genetic and environmental factors (Islam, 2009). Phytoremediation is a technique where hyperaccumulating plants are used for cleanup of metals from metal polluted soils. Metal hyperaccumulating plants can accumulate large amounts of metals in their shoots (Chehregani *et al.* 2009). The high metal tolerant plants can accumulate more than 100-times metals than normal plant species (Sun *et al.* 2006). Metal hyperaccumulating plants can

transport large quantities of metals from the soils into the shoots (Chehregani *et al.* 2009). *Thlaspi caerulescens* and *Brassica juncea* are useful hyperaccumulator plants and their roots can take up metals from polluted soils and translocated these metals into their shoots without inhibiting plant growth (Ishikawa *et al.* 2006). Phytoremediation is an incredible technology and is effective in remediating metal contaminated sites. Its low cost and environmental benefits are applauded by the environmentalists. This technology is also cheaper than traditional methods of remediation (Free Essays, 2003).

The aim of this research is to screen 25 genotypes of tomato for Cd tolerance and Cd accumulation in their shoots. The specific objectives include:

1. Test seed germination responses of 25 genotypes of tomato exposed to various concentrations of Cd.
2. Test 25 tomato genotypes for their ability to accumulate high concentrations of cadmium in their shoots.
3. Elucidate physiological mechanisms of cadmium accumulation in tomato
4. Develop chemical analysis skills using Atomic Absorption Spectrophotometer.

5.2 Materials and Methods

5.2.1 Selection of cultivars

Seeds of 25 genotypes of tomato (M 82, Burke's Backyard, Nash 1, E6203, Tiny Tom, Nash 2, Apollo Improved, Roma VF, Grosse Lisse, San Marzano-2, FLA 456, CLt91t6D4, Red Cherry, Rouge de Marmande, Sweet100F1, Oxheart, Big Beef, CLN2498E, CLN2026D, CLN1621L, Roma, Arka Meghali, CA4, Heir Loom and

Mortgage lifter) were chosen based literature survey. Some seeds were kindly provided by Dr Nanjappa Ashwath and Dr Surya Bhattarai (Central Queensland University) and some were purchased from IGA super market and Bunning's Warehouse.

The reasons for selecting these genotypes includes some of these are commercial cultivars and are known to grow well over a wide range of conditions. Some are breeding lines and are known to possess drought tolerance (FLA456 and Sweet100F1). The use of these genotypes will help identify genotypes suitable for direct use or for utilization in plant breeding programmes.

5.2.2 Preparation of MS media with or without cadmium

The tissue culture media MSsalt (Murashige & Skoog, 1962, Duchefa Scientific Pty.Ltd)) was prepared as shown in Chapter 3, Section 3.1 Cadmium sulphate solution (100 mM) was prepared as shown in Chapter 3.6. Control plants were irrigated with Manutec nutrient solution only. A stock solution containing 100 mM Cd was obtained by dissolving 25.6 g of 3($CdSO_4$) 8H_2O (molecular weight 769.52 g, Sigma-Aldrich Pty.Ltd) in 1000 ml of distilled water. Cadmium stock solution was added to Manutec solution as follows: 0.1 ml of Cd solution was added to 1 L media; for 10 µM, 0.3 ml for 30 µM, 1 ml for 100 µM, 2 ml for 200 µM, 5 ml for 500 µM and 10 ml for 1000 µM cadmium. The pH of the MS media solution was adjusted to 5.3 using pH meter (Orion Research Inc., Model 250A, Boston USA). Therefore, 25 ml of solution was dispensed into clean medium plastic tube (105 mm x 44 mm, polypropylene, non-sterile, Sigma Aldrich Pty.Ltd) using automatic media dispenser (Jencons Scientific Ltd. Boston). All the tubes were sterilised at 121°C and 103 kPa for 21 minutes. After autoclaving, the autoclave door was gently opened and

the tubes were allowed to cool in the autoclave for about 20 minutes to minimise condensation. The pH of the media that contained Cd in MS solution was adjusted to 5.3. All seeds were surface-sterilised with 25% sodium hypochlorite for 5 min in a laminar air flow cabinet before transferring them to medium sized tissue culture tubes (105 mm x 44 mm) containing Cd in MS media. Then all seeds were rinsed 3 times with sterile water. Three tomato seeds were placed on each tube of agar. All tubes were incubated in the controlled environment room with randomised complete block design using three replications. The temperature of growth room was maintained at 25°C and 16 hour light was provided using cool white fluorescent tubes.

5.2.3 Growth parameters

A number of germination and growth parameters were monitored including seed germination percentage, number of leaves, shoot height, shoot weight as well as shoot Cd concentrations. Morphological symptoms of plants in response to Cd treatments were also recorded. Seed germination was determined by counting the number of germinated seedling once a week for 3 weeks. Shoot height was measured using a ruler and the shoot tissues were analysed for Cd concentration. The tissue Cd concentration was determined using Atomic Absorption Spectrophotometer (AAS).

5.2.4 Cadmium analysis of plants

After five weeks, the seedlings of each tube were harvested. The shoots were separated into leaves and stems before drying in the oven at 72ºC for 3 days. The dry weights of the shoots were recorded. Plant samples were ground into a fine powder (<1.5 mm) using Mikro-Feinmuhle-Culatti (MFC) grinder. The powder was digested with 2 ml of nitric acid (HNO_3) and one drop of hydrogen peroxide (H_2O_2). The

plastic tubes were placed over night in the fume hood. On the following morning, 3 ml of Milli-Q water was added and was placed in a water bath (Grant Instrument, Cambridge Ltd) maintained at 70ºC for 4 hours. After digestion, the volume of the tube was made up to 10 ml using Milli-Q water. The tube was centrifuged at 15,000 g for 15 min and the supernatant was used in the analysis. The concentrations of Cd in the digested samples were determined using AAS spectrophotometer.

5.2.5 Statistical analysis of data

Analysis of variance was performed using Genstat version 13 following testing: the data for outliers and homogeneity of error variances. Standard errors of means or least significant differences (lsd) were used for comparing the means.

5.3 Results

5.3.1 Effect of cadmium on seed germination

Most of the tomato seeds germinated at all Cd levels after two weeks. A hundred percent seed germination was found in 15 of the 25 tested genotypes. Seeds did not germinate in genotype CLN1621L at 500 and 1000 µM Cd (Fig. 5.1). There was little difference between the genotypes in seed germination. Only in genotype CLN1621L, seed germination was affected by Cd at higher concentrations.

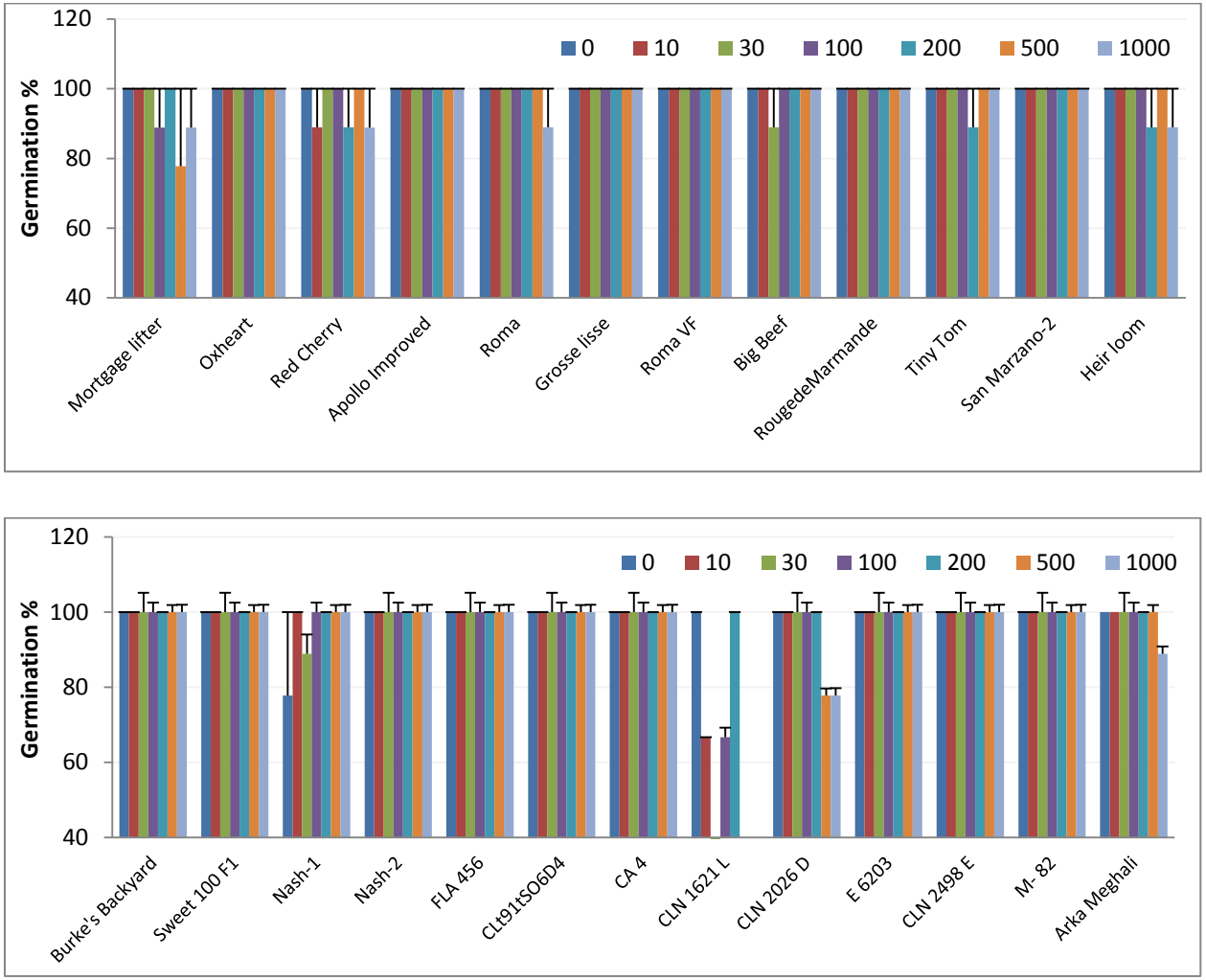


Figure 5.1 Seed germination percentage in 25 genotypes of tomato established in tissue culture media containing 0 to 1000 µM Cd. Bars represent SE (n=3).

5.3.2 Effect of cadmium on shoot height

The responses to cadmium concentrations in the media varied between the genotypes. Overall, lower concentrations (10, 30, 100 µM) of Cd slightly improved height growth of many genotypes and higher Cd concentrations (1000 µM) significantly reduced seedling height in all genotypes (Fig. 5.2).

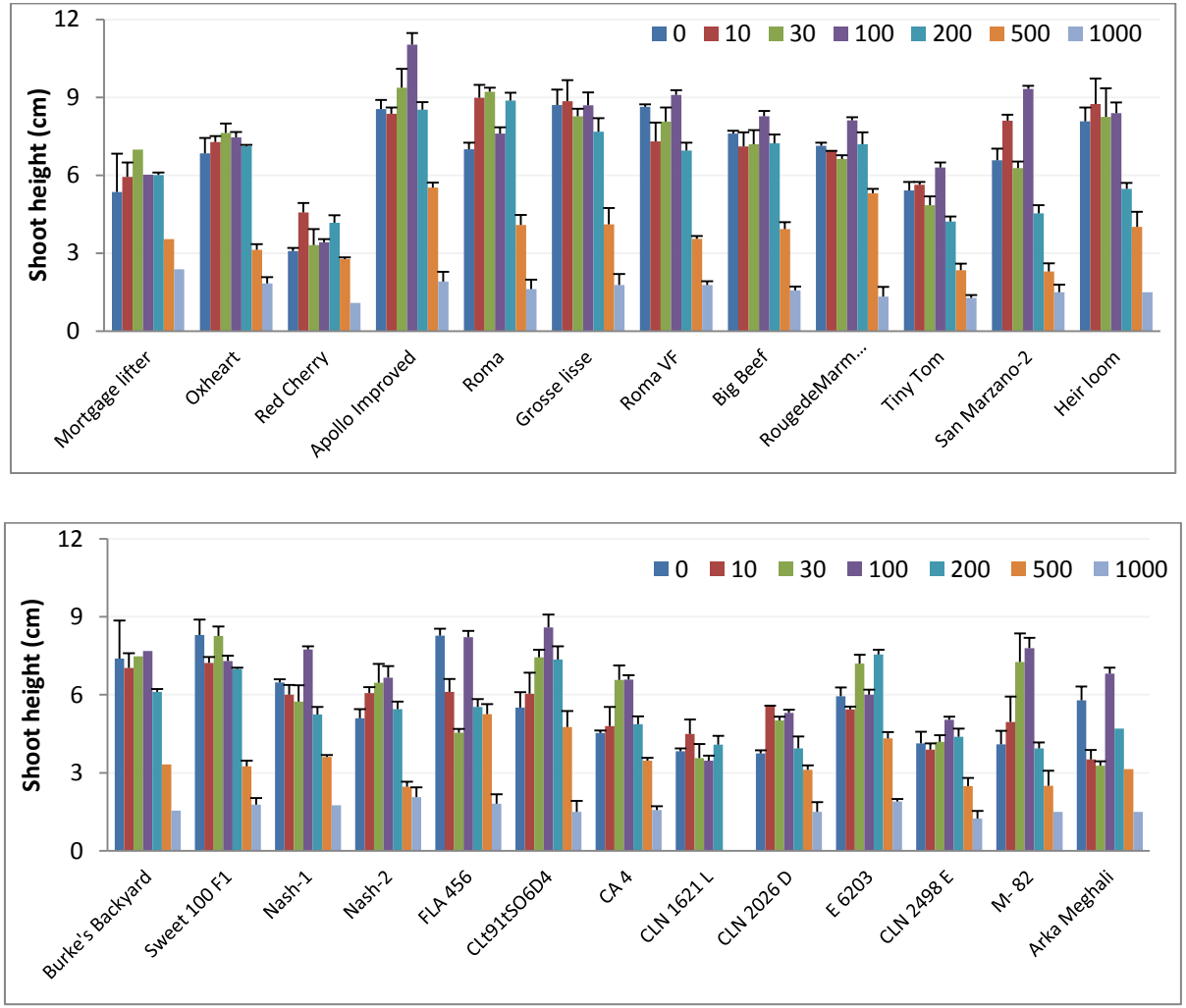


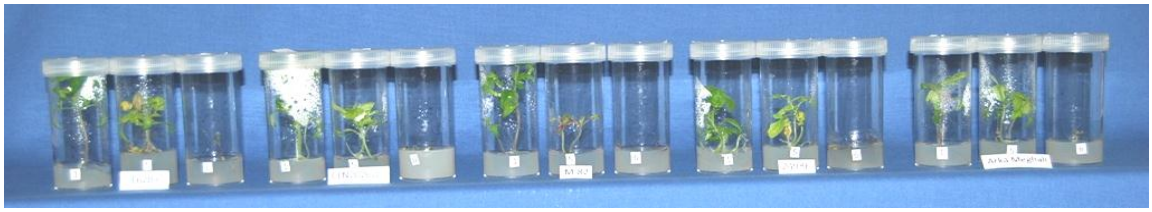
Figure 5.2 Shoot height of 25 genotypes after 5 weeks of exposure to Cd.
Bars represent SE (n=3).

A

B

C

D



E

Plate 5.1 Photographs showing growth of tomato seedlings treated with 1=control, 5=500 µM Cd and 6=1000 µM Cd, (A): genotypes- Roma, Apollo Improved, Red Cherry, Oxheart and Mortgage lifter, (B): genotypes-Burke's Backyard, Big Beef, Rouge de Marmande, Grosse Lisse and RomaVF, (C): genotypes- FLA456, Sweet 100F1, Tiny Tom, San Marzano-2 and Heir loom, (D): genotypes- Nash2, Nash1, CA4, CLt91t6D4 and CLN1621L, (E): genotypes- E6203, CLN2026D, M82, 2498E and Arka meghali.

5.3.3 Effects of cadmium on number of leaves

The numbers of leaves were severely affected by high Cd concentrations (500 and 1000 µM). At lower Cd levels (10, 30, 100 or 200 µM) the leaf number per plant were higher in many genotypes compared to control after 5 weeks of exposure to Cd. The highest number of leaves was observed in genotypes Nash1 and Sweet100F1 at low Cd levels. The lowest number of leaves was found in Tiny Tom and San Marzano-2 at high Cd levels (Fig. 5.3). Overall, unusual genotypes that means seeds did not germinate or seedlings did not grow well, no leaves were found at high Cd levels. There was a significant difference between the genotypes and amongst the Cd concentrations in the number of leaves produced. The leaf numbers were drastically reduced at 500 and 1000 µM Cd in most of the genotypes produced at different Cd levels.

Seedlings did not survive in 13 of the 25 genotypes, and those which produced the leaves at these concentrations also have very few leaves (Fig. 5.3).

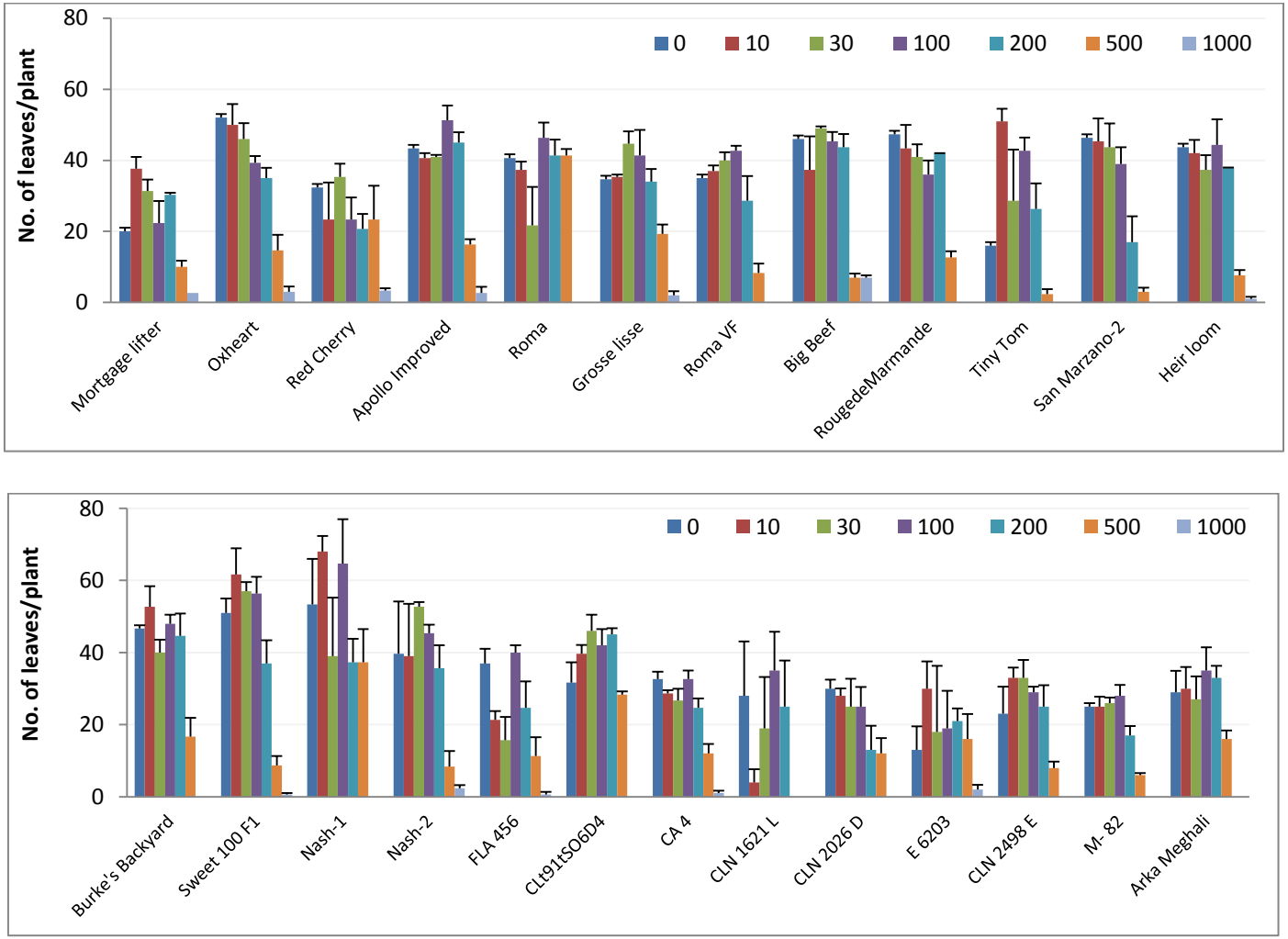


Figure 5.3 No. of leaves in 25 genotypes in *in vitro* treated with control, 10, 30, 100, 200, 500 and 1000 µM Cd. Bars represent SE (n=3).

5.3.4 Effect of cadmium on leaf dry weight

Leaf dry weight was very low in genotypes Oxheart, Red Cherry, CA4 and Mortgage lifter. Highest leaf weight was found in Apollo Improved and CLt91t6D4 (Fig. 5.4) compared to control. The leaf dry weight increased in 100 µM Cd treatments in genotypes Grosse Lisse, RomaVF, Heir Loom, Nash2, M82 and Arka Meghali. However, genotypes Roma, Rouge de Marmande, Roma VF, Tiny Tom, San Marzano-2, Heir Loom, Burke's Backyard, Nash1, CLt91t6D4, CLN1621L, CLN2026D, CLN2498E, M 82 and Arka Meghali were highly sensitive to highest Cd concentrations 1000 µM, and the seedlings did not grow well and they were not able to remain erect after two weeks of exposure to Cd. The leaf dry weight differed significantly ($P<0.05$) between the genotypes and the Cd concentrations.

5.3.5 Effect of cadmium on stem dry weight of tomato

A significant interaction between the genotypes and the Cd concentrations suggested that the response of genotypes varied with the Cd levels (Table 5.2). The lower stem dry weight was found in genotypes Mottage lifter, Oxheart, Red Cherry, Big Beef, Burke's Backyard, CA4 and CLN1621L. Stem dry weight was higher in Apollo Improved, RomaVF, San Marzano-2, Nash1, Nash2, FLA456 and CLt91t6D4. At 100 µM Cd stem dry weight increased in genotypes Oxheart, Apollo Improved, Roma, RomaVF, Big Beef, Nash2, CLN2026D, M82 and Arka Meghali as compared to control. Likewise at stem dry weight increased at 30 µM in San Marzano-2 at and at 200 µM in CLt91t6D4. Seedlings did not grow well and fallen at 1000 µM Cd in most genotypes except Nash2 and Mortgage Lifter (Fig. 5.5).

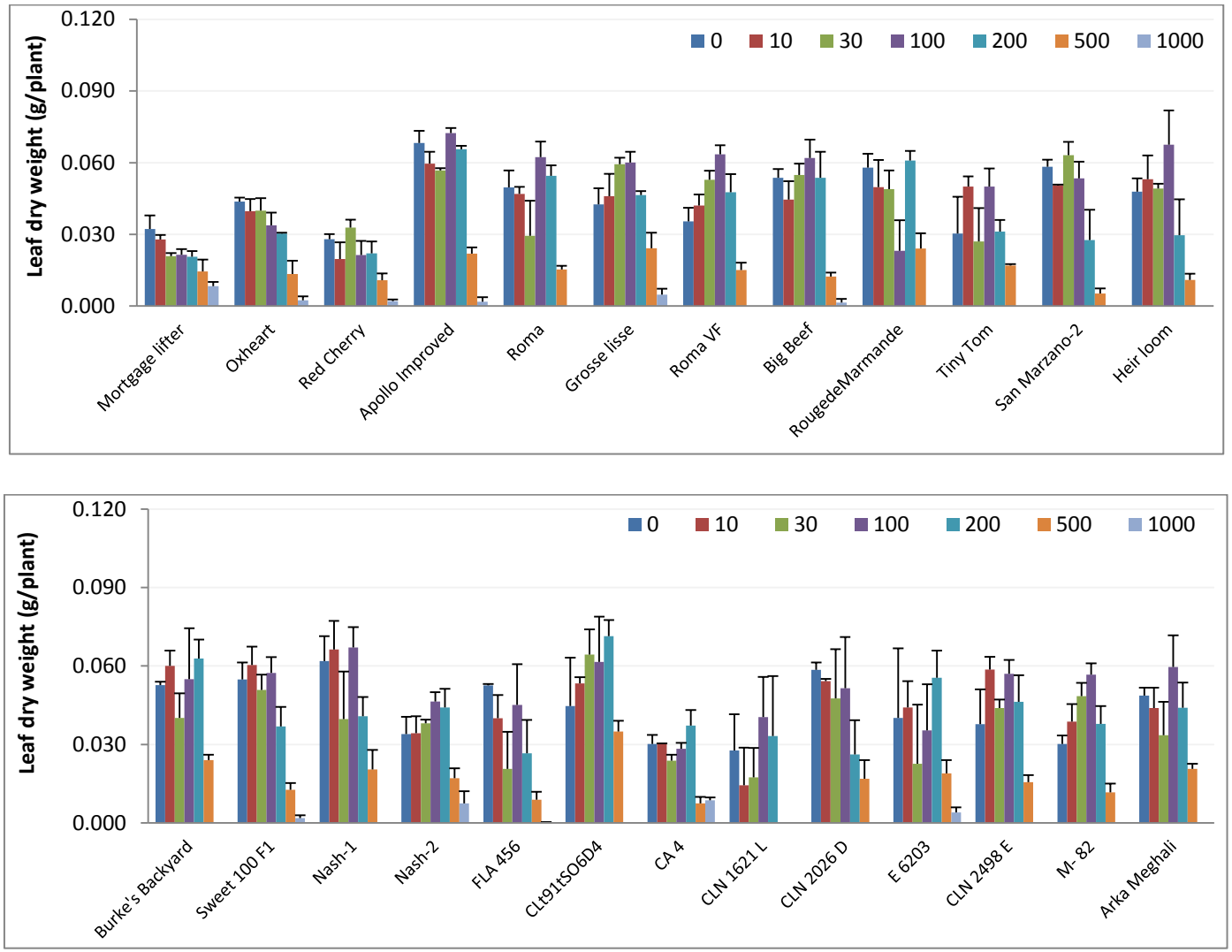


Figure 5.4 Leaf dry weight of 25 genotypes after 5 weeks.
Bars represent SE (n=3).

5.3.6 Foliar symptoms

At higher Cd concentrations, plants showed Cd toxicity symptoms on leaves. These included yellowing of the leaf blade, black spot, and necrosis and leaf drop at 100, 200, 500 µM Cd. These symptoms were rare to occur at 0, 10, 30 µM Cd. Seedlings were fallen and dead in many genotypes at 1000 µM Cd (Plate 5.2).

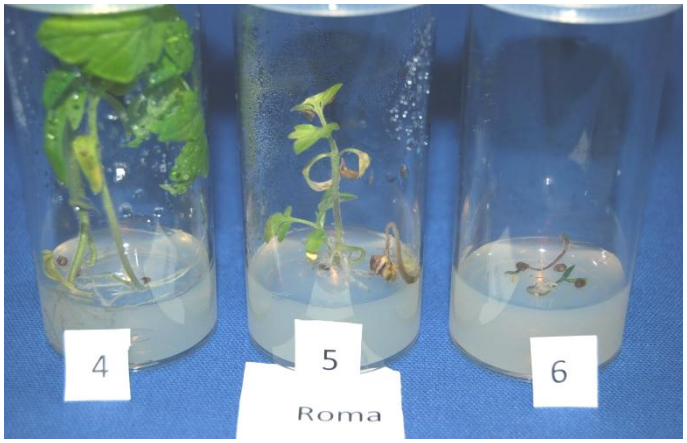


Plate 5.2 Cadmium toxicity symptoms in Roma treated with 4=200, 5=500 µM and 6=1000 µM Cd

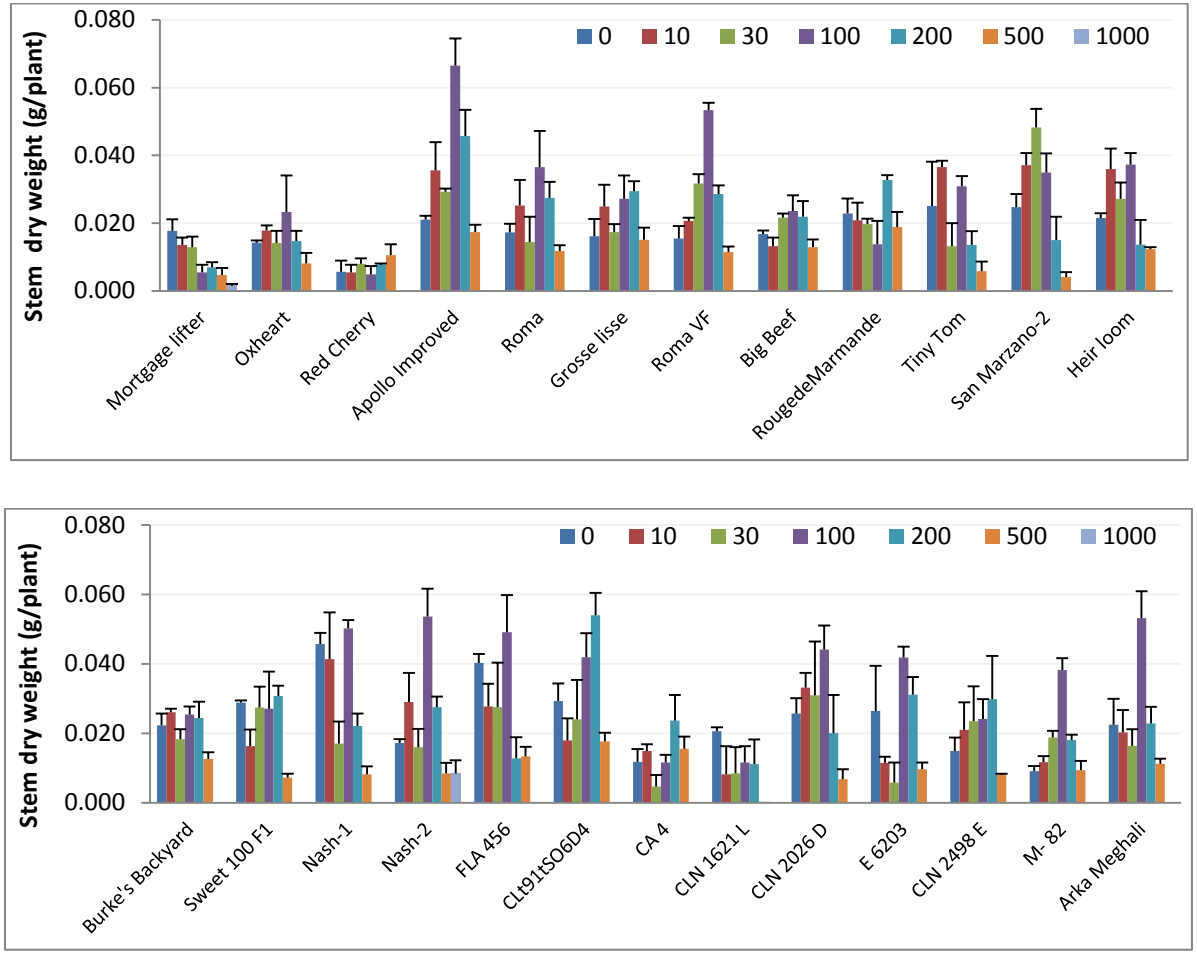


Figure 5.5 stem dry weight of 25 genotypes after 5 weeks of exposure to various Cd concentrations. Bars represent SE (n=3).

5.3.7 Cadmium concentration in tomato leaves

Cadmium concentrations of the leaves increased as the media concentrations increased. The highest Cd concentration was observed in Nash2 (1412 µg/g) and Grosse Lisse (925 µg/g) at 1000 µM Cd after five weeks of exposure to Cd (Fig. 5.6). The lowest Cd accumulation was found in Big Beef (50 µg/g) treated with 1000 µM Cd. The 10 of 25 tomato genotypes, viz. Nash2, Apollo Improved, Grosse lisse, Nash1, E6203, Red Cherry, Sweet 100F1, Oxheart, Big Beef and CA 4 were tolerant to Cd and accumulated high Cd concentrations at 1000 µM. However, 15 of 25 genotypes viz., Roma, RomaVF, Rouge de Marmande, Tiny Tom, San Marzano-2, Mortgage lifter, Heir Loom, M 82, Burke's Backyard, FLA456, CLt91t6D4, CLN1621L, CLN2026D, CLN2498E, and Arka Meghali were highly sensitive to 1000 µM Cd. The leaf Cd concentration significantly ($P<0.001$) differed with the genotype and the media Cd concentrations (Table 5.1).

5.3.8 Leaf cadmium uptake in tomatoes

The leaf Cd uptake was calculated based on Cd concentration and dry weight after 5 weeks. The genotypes were grouped into three categories (Table 5.2) based on Cd uptake. The 6 genotypes Rouge de Marmande, Big Beef , Roma, Red Cherry , Oxheart and CLN1621L had low uptake rates (<10 ug/plant), while the genotypes in the 12 genotypes FLA456 , RomaVF, Tiny Tom , Heir loom , Arka Meghali, CLN2498E, E6203, CLN2026D, Nash2, CA4, Apollo Improved and M82 had moderate (10-20 ug/plant) levels of uptake. The high uptake category (20-35 ug/plant) included 6 genotypes CLt91t6D4, Grosse Lisse, Nash1, Burke's Backyard, Sweet100F1 and San Marzano-2. The uptake rate differed significantly between the

Cd concentrations and the genotypes and there was a significant interaction between the treatment and the genotypes (Table 5.2).

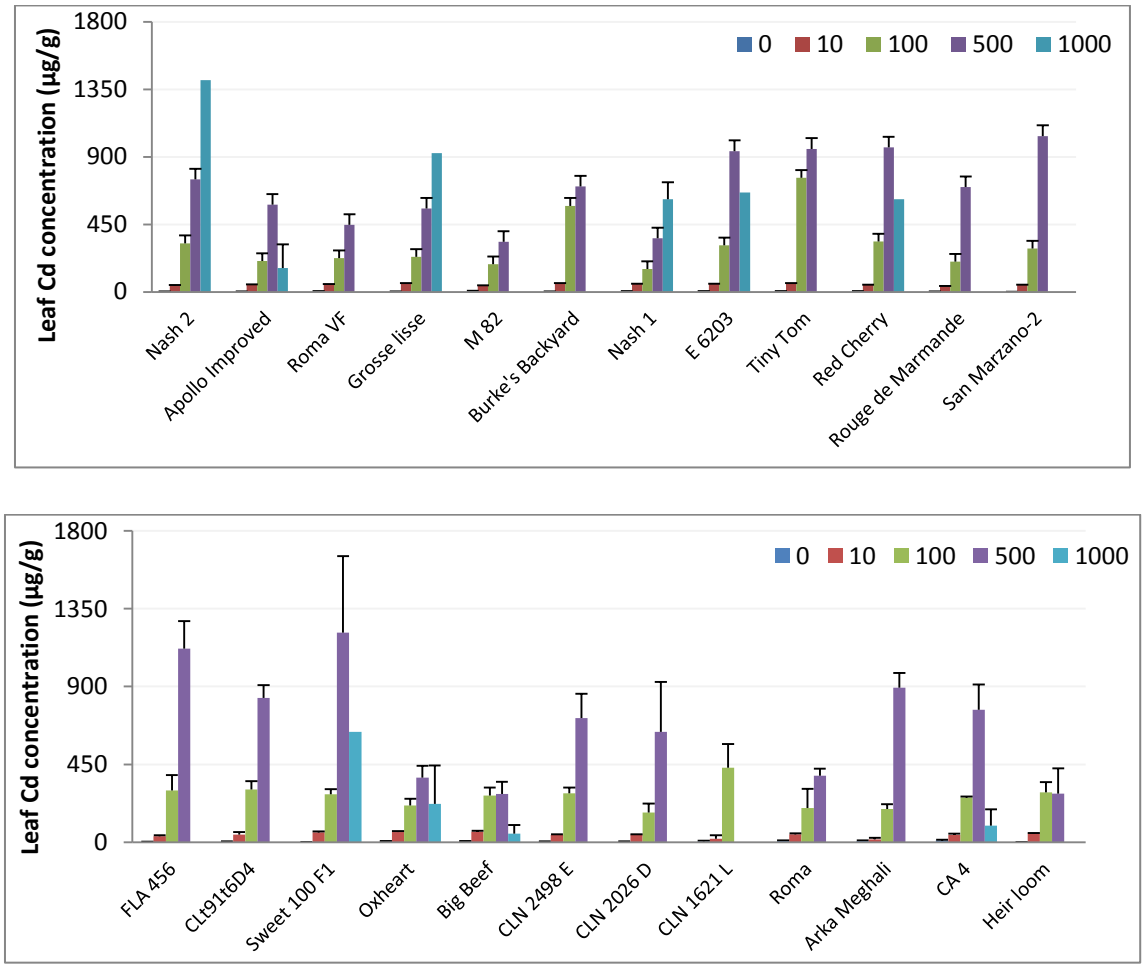


Figure 5.6 Cd concentration (µg/g) in 25 genotypes treated with control, 10, 100, 500 and 1000 µM after 5 weeks of exposure to Cd. Bars represent SE (n=3).

Table 5.1 Uptake of Cd (low, moderate and high) in 25 genotypes of tomato treated with control, 10,100,500 and 1000 µM Cd

Low (< 10 µg/plant)	**Moderate(10- 20) µg/plant**	**High (20-35) µg/plant**
Rouge de Marmande (9.9)	FLA456 (19.5)	CLt91t6D4 (32.4)
Big Beef (9.9)	RomaVF (19.4)	Grosse Lisse (31.8)
Roma (9.3)	Tiny Tom (19)	Nash1 (31.5)
Red Cherry (8.2)	Hire loom (18.9)	Burke's Backyard (27.4)
Oxheart (6.9)	Arka Meghali (17.8)	Sweet100F1 (26)
CLN1621L (0.49)	CLN2498E (17)	San Marzano-2 (21)
	E6203 (16)	
	CLN2026D (15.6)	
	Nash2 (15.4)	
	CA4 (12.5)	
	Apollo Improved (12.4)	
	M 82 (11.3)	

Table 5.2 *P* values from ANOVA for 25 genotypes

	Df	**G%**	**df**	**Pt ht**	**df**	**no. L**	**SDW**	**df**	**LDW**	**Df**	**L Cd conc**	**df**	**L Cd uptake**
Genotype	**25**	**<.001*** **	**25**	**<.001*** **	**25**	**<.001*** **	**<.001*** **	**25**	**<.001*** **	**23**	**<.001*** **	**23**	**<.001*** **
Genotype*treatmentt	**144**	**0.057***	**142**	**<.001*** **	**130**	**<.001*** **	**<.001*** **	**129**	**0.021***	**76**	**<.001*** **	**77**	**<.001*** **

Df (n-1)=degrees of freedom, G%=germination percentage, Pt ht=plant height, SDW=stem dry weight, LDW=leaf dry weight, LCd conc=leaf Cd concentration, LCd uptake=leaf Cd uptake.

5.4 Discussion

The effects of Cd on seed germination, shoot height, shoot weight, leaf Cd concentration and Cd accumulation was assessed in tissue culture media using 25 genotypes of tomato and seven concentrations of Cd (0, 10, 30, 100, 200, 500 and 1000 µM) over 5 weeks.

Germination data showed very little differences between the genotypes except at very high Cd concentration. However, leaf symptoms, shoot weight and root weight data revealed significant differences between the genotypes. Lefevre *et al*. (2009) reported inhibited germination and reduced fresh weight of roots and shoots in Mediterranean leguminous shrub *Dorycnium pentaphyllum* treated with 1000 µM Cd. In contrast, more than 90% of seed germination was found in lettuce, broccoli, tomato and radish at 0-1024 µM Cd (Salvatore *et al*. 2008).

In this study, the treatment with lower Cd levels increased shoot height of many genotypes but at higher Cd concentrations (500 and 1000 µM), reduced seedling height was noted. Cho, (2004) found that the shoot height of tomato cv. Seokwang was increased at 10 µM Cd but it decreased at 100 µM Cd. Shoot and root length of tomato declined at 10 µM Cd after 4, 8 and 12 days of exposure (Deef, 2008). Similarly, shoot growth reduction has been reported for Cu, Zn and Mn at various concentrations (Gatti, 2008). The shoot length of the cultivar *Ailanthus altissima* decreased at 60, 120, 240 and 480 µM Zn as compared to the control. The shoot length decreased in cultivar *Ailanthus altissima* in 1600 µM Mn but it increased at 200 µM Mn when compared to control (Gatti, 2008). The shoot length decreased at 100, 250 and 1000 µM Cu in cultivar poplar (*Populus tremula*) when compared to

the control. The shoot length decreased in poplar (*Populus tremula*) at 1000 and 2000 µM Pb compared to control (Bojarczuk 2004).

In the present study, the number of leaves increased at lower concentrations and decreased at higher concentrations. Lower concentrations of salt (Aswathappa and Bachelard 1986, Aswathappa 1988) or heavy metals (Deef, 2008) are also known to enhance plant growth. However in apple-tree rootstock M9 and B396 treated with 5 µM Cd the number of green leaves reduced as compared to the control. No green leaf was observed in both cultivars at 2000 µM Cd (Sakalauskaite *et al*. 2006).

In this study, the leaf dry weight decreased in all genotypes at 500 and 1000 µM Cd but it increased at lower concentrations in Roma, Grosse Lisses, RomaVF, Big Beef, Tiny Tom, Heir Loom, Nash2, CLt91t6D4, CLN1621L, CLN2498E, M82 and Arka Meghali. The stem dry weight increased in most of the genotypes at low Cd level but decreased in Mortgage lifter. Leaf dry growth was inhibited in tomato Micro-Tom at 500 and 1000 µM $CdCl_2$ but gradual increase in Cd concentration exhibited growth rates partially identical to the control after 75 days of growth (Grato *et al.* 2008). Leaf, stem and root dry weight of tomato cv. Hezuo 903 and Jiang-shu 14 decreased at 10 µM Cd (Dong *et al.* 2005). The leaf and root dry weight decreased in tomato plants treated with 50 µM Cd but not at 5 µM Cd (Quariti *et al.* 1997). The leaf weight of lettuce and spinach was decreased at 0.32 µM Cd (McKenna *et al.* 1993). Leaf, stem, root and bulb weight of *Arum* was decreased at 10 to 50 µM Cd. Root and shoot weight of radish was also decreased at 10 µM Cd. Leaf, stem and root weight of water spinach was decreased at 1.5 to 10 µM Cd (Kashem *et al.* 2008). The biomass inhibition was observed in tomato at 50 µM Cd (Ammar *et al.* 2008). The leaf dry weight decreased in tomato cultivar Palace at 200 and 400 µM Cd

(Inouhe *et al*. 1991). The fresh weight and dry weight decreased in cultivar peach rootstock at 100 µM $CuSO_4$ after 20 days of exposure (Lombardi and Sebastiani 2004). The growths rate were inhibited and reduced dry weight of tomato were recorded at 150 and 200 µM Cd after 8 days. The dry weight of adzuki bean was reduced at 10 µM Cd. The adzuki bean cells were much more sensitive to Cd than tomato cells (Inouhe *et al*. 2000). In contrast, the fresh weight and dry weight of cucumber (*Cucumis sativus*) increased at 200 µM Cd after 24 days of exposure (Gzyl and Gwozdz 2005).

In this experiment Cd exposed plants showed purpling, chlorosis and black spots and no genotype specific or Cd specific symptoms were found. Lopez-millan *et al. (*2009) observed symptoms of leaf necrosis, chlorosis, leaf drop and wilting in most genotypes that were treated with high Cd. Biomass production was inhibited by Cd at concentrations ranging from 0 to 50 µM. Cadmium also reduced leaf chlorophyll content of tomato (Ammar *et al.* 2008). In tomato treated with 2 µg/ ml Cd, chlorosis was found in split-root and intact plants (Smith and Brenna 1983). Toxicity symptoms of chlorosis, white spots and old leaves rolling were found in young leaves of radish and water spinach at 1.5 and 10 µM Cd (Kashem *et al.* 2008). Leaf necrosis was observed in tomato when the seedling was exposed to 1 µM Cd (Dong *et al.* 2005).

In the present study, 10 of 25 tomato genotypes were tolerant to Cd and they accumulated high concentrations of Cd at 1000 µM. However 15 of the 25 genotypes were highly sensitive to Cd at 1000 µM. Grato *et al.* (2008) reported that the cadmium accumulation increased in tomato cv. Micro Tom at 1000 µM $CdCl_2$ after 75 days. In potato, cultivar Macaca was contained higher Cd than cultivar Asterix at

100, 200, 300, 400 and 500 µM Cd (Goncalves *et al*. 2009). The Cd tolerant cell line was higher when compared to Cd sensitive cell line in cucumber treated with 100 and 200 µM Cd after 9 days of exposure to Cd (Gzyl and Gwozdz 2005*).*

In the present experiment, the low leaf Cd uptake was observed in 6 genotypes, the medium Cd uptake was determined in 12 genotypes and high Cd uptake was found in 6 genotypes. Cadmium uptake (3.2 mg/plant) increased in the shoots of *Sedum alfredii* at 400 µM Cd but decreased (2.9 mg/plant) at 200 µM Cd (Yang *et al.* 2004). The highest uptake of Cd was found in tobacco with Cd 300 mg L^{-1} (Vasiliadou and Dordas 2009). Cadmium uptake of shoots increased in *Sedum jinianum* treated with 100 and 200 µM Cd (Xu *et al.* 2009). The high Cd uptake was found in tomato cultivar Palace treated with 400 µM Cd (Inouhe *et al*. 1991).

Comparison of genotypes for Cd uptake will help to determine which of the tested genotyes will transport high levels of Cd into shoots. This information is extremely important, as this will help estimate the time taken to remove Cd from contaminated sites to a pre-determined level or to the level required by the regulatory authorities. This information will also be necessary as the tissue concentration or biomass accumulation on their own will be inadequate to determine the efficiency of phytoremediation.

In conclusion, the results showed 15 of 25 tomato genotypes were tolerant to high Cd toxicity and were able to germinate well in the presence of Cd. However, after seed germination, 15 of 25 tomato genotypes became hypersensitive to high Cd concentrations (1000 µM). All 25 tomato genotypes were tolerant to low Cd level, but they were sensitive to 500 and 1000 µM Cd. Ten of the twenty genotypes of tomato were tolerant and accumulated highest Cd in their leaves. These genotypes

will have great potential for use in phytoremediation of Cd contaminated sites. The highest Cd uptake was found in 6 of 25 genotypes, and they include CLt91t6D4, Grosse Lisse, Nash1, Burke's Backyard, Sweet100F1 and San Marzano-2.

Chapter 6

Cadmium uptake and shoot distribution by four genotypes of tomato grown in sand culture

6.1 Introduction

Cadmium is one of the most toxic heavy metals and it is easily absorbed by plant roots and subsequently distributed to aerial parts of the plants (Treder and Cieslinski 2005). Cadmium pollution can occur in air, water and soil causing environmental contamination and phytotoxicity to plants (Ruiz *et al.* 2009). The application of industrial effluents for irrigation in horticultural farms can result in accumulation of these metals in the soils. This metal pollution could affect food quality and safety (Arora, 2008). Exposure of plants to Cd can result in membrane distortion, retardation of enzyme activity and inhibition of photosynthesis and imbalance of nutrient uptake. The synthesis of glutathione prevents Cd toxicity in plant tissues (Rodriguez-Celma *et al.* 2010). The use of phosphate fertilizers and pest control chemicals in crop production may lead to serious health problems in humans via Cd contamination (Pandey and Pendy, 2009). Cadmium can cause renal dysfunctions and loss of minerals in the bones of human beings, even if they consume only a small amount of cadmium in their diets (Salt *et al.* 1995). Urban and industrial waste water contain toxic metals that can cause soil pollution which in turn become phytotoxic to plants. When animals and humans consume these contaminated plants, health problems can occur (Topcuoglu, 2005). Cadmium accumulation and translocation to aerial parts of plants are determined by soil fertility and quality, soil pH and other physiological factors (Treder and Cieslinski, 2005). The natural agricultural soils contain Cd ranging from 0.1 to 0.5 mg/kg. Molten rocks and

metamorphic rocks contain small amounts of Cd (0.02 to 0.2 mg/kg). The sedimentary rocks contain Cd from 0.1 to 25 mg/kg. Coal, oil and natural gas contain 0.5 to 1.5 mg/kg. However, some fertilizers contain 10 to 200 mg/kg Cd (International Cadmium Association 2010). In Australia, the natural soil environment contains less than 0.1 to 0.5 mg/kg or about 0.1 to 0.7 kg Cd/ha in the top soil layers (Vege Notes, 2003).

More than 400 species of metal hyperaccumulator plants have been identified in the whole world. These plants occur in metal-rich soils and they accumulate high amount of metals in their shoots (Sun *et al.* 2006). Soil acidity and concentrations of metals in the soil are associated with metal uptake of the plant (Moral *et al.* 2002). If the soil contains more than 10 mg/kg Cd, it could cause toxicity to plants. This may include inhibition of seed germination and seedling growth, reduced biomass production, reduction in chlorophyll content, reduced respiration and absorption of nutrients and water (Gabrijel *et al.* 2009). Human beings consuming leafy vegetables containing elevated levels of Cd are more likely to accumulate cadmium throughout their bodies compared to those who eat non Cd contaminated vegetables (Wang *et al.* 2007). Hyperaccumulating plants can be used to remove metals from metal contaminated soils because they are able to transport metals from the soils to their aerial parts in large quantities (Bert *et al*. 2003). Heavy metal accumulation by a plant is assessed by its biological absorption coefficient (BAC) which is the ratio of Cd concentrations in the shoots to that in the growth medium. Plants with high BAC (>1) are suitable for phytoremediation (Vamerali *et al*. 2010).

The aim of this research is to compare Cd accumulating patterns of four genotypes of tomato that were selected from previous tissue culture experiments, based on varying degrees of Cd accumulation in their shoots. The specific objectives were to:

1. Determine the patterns of Cd accumulation between the four genotypes of tomato grown in a glasshouse in sand culture.
2. Compare the results of this experiment with those conducted in tissue culture, particularly for Cd concentrations of the shoots.
3. Elucidate growth and cadmium distribution patterns of the four genotypes of tomato, in response to varied times of exposure to Cd (3, 5, 7 and 9 weeks after exposure to Cd).

6.2 Materials and Methods

6.2.1 Selection of tomato genotypes

Seeds of four tomato genotypes, viz., Big Beef and Burke's Backyard (produced by Yates) and Tiny Tom and Grosse Lisse (produced by Mr. Fothergill's) were selected from the experiments described in Chapter 5 based on their varied patterns of Cd accumulation. The seeds were purchased from IGA Super Market and Bunning's Warehouse. The reasons for selecting these genotypes are:

1. Big Beef: Low Cd accumulator (see Chapter 5)
2. Tiny Tom: Medium to low Cd accumulator
3. Burke's Backyard: Medium to high Cd accumulator
4. Grosse Lisse: High Cd accumulator

6.2.2 Pot culture

Plants were grown in a glasshouse using sixty small plastic polyethylene pots (12 x 9 x 12.5 cm) containing sand as the growth medium. The pots were set up as shown in Plate 6.1.

Plate 6.1 Sand cultures of four genotypes of tomato grown in a glasshouse

6.2.3 Collection of sand

Washed river sand was procured from a local landscape supplier. The sand was dried in a dryer at 60˚C for two days. Dried sand was filled into each pot and weighed (1.5 kg). The pots were placed on a glasshouse bench that was lined with a thick plastic sheet to prevent Cd solution leaking from the pots. Saucers were placed under the pots to ensure that excess water was collected in the saucer and was allowed to be reabsorbed by the plant. Cadmium stock solution (100 mM) was prepared as described in Chapter 3.6. Ten seeds of each genotype were sown in each pot. To each pot 200 ml of reverse osmosis (RO) water was poured until the seeds were

germinated. After 7 days of germination, five Cd treatments, viz. 0, 30, 200, 500 and 1000 µM Cd were imposed using three replications. Preparation of hydroponics nutrient solution with or without Cd is described in Chapter 3.7. After seed germination, Manutec (Part 1=N.P.K.7.6/3.1/18.2 + Part 2; Trace elements=calcium nitrate) hydroponics (Manutec Garden Care Products) nutrient solution with or without Cd was added to all pots at 200 ml/pot during the first week and 120 ml/pot during the second week. The experiment was run in a glasshouse which was maintained at a temperature of 25-32°C using an evaporative cooler. The experiment was replicated twice.

6.2.4 Growth Parameters

Seed germination, plant height, leaf number, stem and leaf dry weight, cadmium concentrations in the leaves and stems were recorded at 3, 5, 7 and 9 weeks after imposing cadmium treatments. Morphological symptoms of plants in response to Cd exposure were also recorded. The shoot tissues were analysed for Cd concentration as described in Chapter 5. The tissue Cd concentration was determined by Atomic Absorption Spectrophotometer.

6.2.5 Tissue analysis for the cadmium

After three weeks of exposing the seedlings to Cd, three randomly selected seedlings from each pot were harvested and were washed with demineralised water. The shoots were separated into leaves and stems, and the leaves were counted before drying in the oven at 72 °C for 3 days, and recording the dry weights. Plant samples were ground into a fine powder (<1.5 mm) using Mikro-Feinmuhle-Culatti (MFC) grinder. The powder (0.3 g) was placed in a plastic (PPTR) tube, and 2 ml of concentrated nitric acid (HNO_3) was added along with one drop of hydrogen

peroxide (H_2O_2). The plastic tubes were placed in a fume hood overnight. On the following morning, 3 ml of Milli-Q water was added and the tubes were placed in a water bath maintained at 70°C for 4 hours. After digestion, the volume of the tube was made up to 10 ml using Milli-Q water. The tube was centrifuged at 15,000 gfor 15 minutes, and the supernatant was analysed for Cd using Atomic Absorption Spectrophotometer. The seedlings were harvested at 3, 5, 7 and 9 weeks after exposing to Cd treatments. At each harvest, shoots of three seedlings were removed from each pot. The shoots were rinsed in demineralised water and dried in an oven at 72ºC for 3 days. The dried shoots were ground using a MFC grinder and the powder was used in chemical analysis.

6.2.6 Biological Absorption Coefficient (BAC)

Biological Absorption Coefficient (BAC) was calculated according to Malik *et al*. (2010), as the ratio of cadmium concentration in the shoots to that in the sand culture as:

$$\text{BAC (µg/g/plant)} = \frac{\text{Metal concentration in the shoots}}{\text{Metal concentration in the growth media}}$$

6.2.7 Statistical analysis

Analysis of variance was performed using Genstat version 13 following testing the data for normality, outliers and homogeneity of error variances. Standard errors of means or least significant differences (lsd) $P<0.05$ were also used when the ANOVA was not significant.

6.3 Results

6.3.1 Effects of cadmium on seed germination

Seed germination percentage decreased slightly from 100% (control) to 97% at 1000 µM Cd in Big Beef, at 30 and 500 µM Cd in Tiny Tom, at 30, 200 and 1000 µM Cd in Burke's Backyard and at 200 and 500 µM Cd in Grosse Lisse. Overall, Cd had negligible effect (<5% reduction) on seed germination (Fig. 6.1).

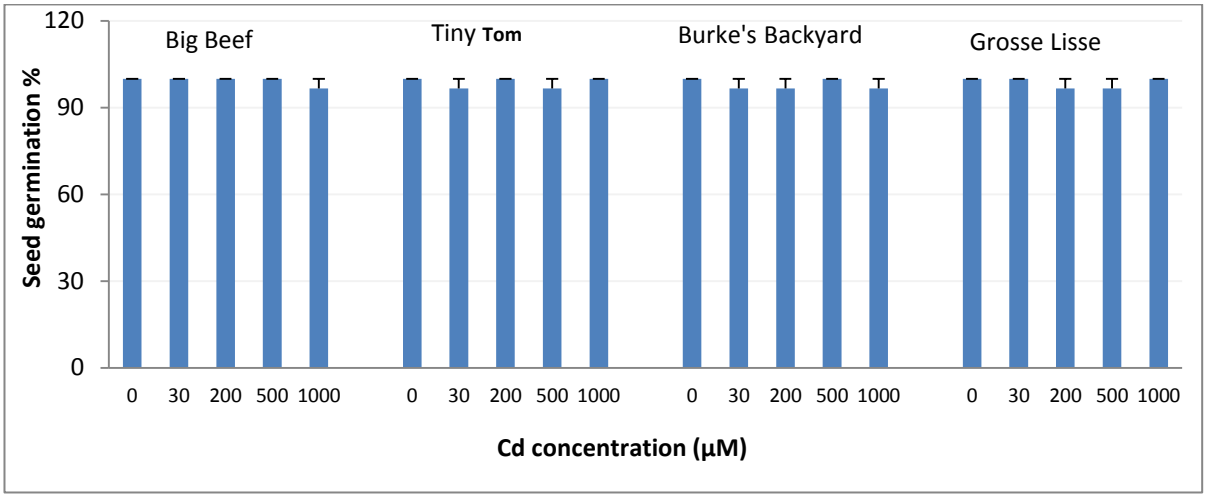


Figure 6.1 Seed germination percentage of 4 genotypes of tomato in sand culture treated with 0, 30, 200, 500 and 1000 µM Cd.
Bars represent SE (n=3).

6.3.2 Effect of cadmium on plant height

Cadmium did not affect plant height except in Tiny Tom at 1000 µM Cd (Fig. 6.2).There were no significant differences between the four genotypes in their response to Cd treatment.

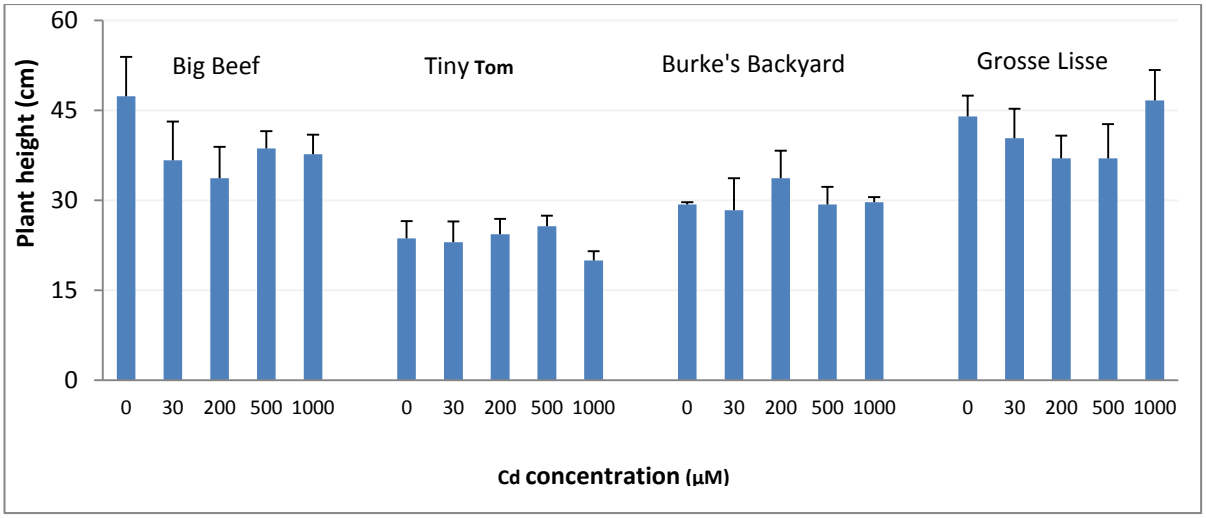


Figure 6.2 Plant height of 4 genotypes of tomato treated with 0 to 1000 µM Cd for 9 weeks. Bars represent SE (n=3).

6.3.3 Effects of cadmium on number of leaves

In genotype Tiny Tom, leaf number increased with an increase in media Cd concentration (1000 µM) compared to the control (Fig. 6.3). However there were little differences between the treatment in other three genotypes Big Beef, Burke's Backyard and Grosse Lisse.

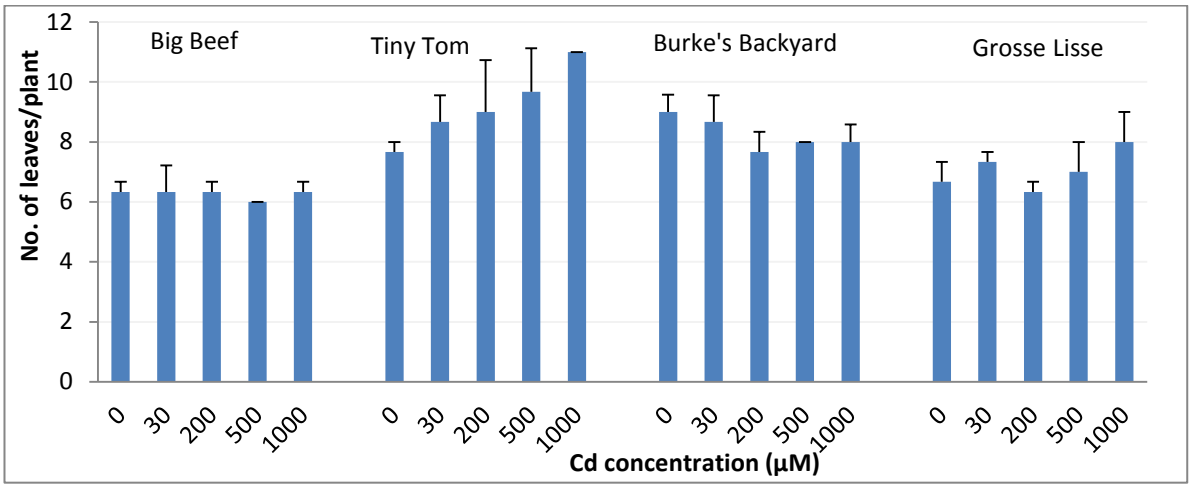


Figure 6.3 Number of leaves in 4 genotypes of tomato after 9 weeks of exposure to various concentrations of Cd.
Bars represent SE (n=3).

6.3.4 Effects of cadmium on leaf dry weight

It must be noted that three seedlings were removed at each harvest, and when the variation between seedlings were found high then this variation must be taken into account while comparing different types of Cd treatments. In Big Beef, the leaf dry weight was similar between the Cd treatments at week 3, slightly decreased with Cd at week 5, and slightly increased with Cd (up to 500 µM Cd) in weeks 7 and 9. Amongst all Cd treatments, 1000 µM Cd had the most effect on plant growth. This genotype grew fast and was affected by high Cd (500 µM Cd) after 7 and 9 weeks (Fig. 6.4).

In Tiny Tom, the leaf dry weight was not different amongst the Cd treatments after 3 and 5 weeks but decreased after 7 weeks, as the Cd concentration increased at 1000 µM Cd. Leaf dry weight was high in 200 µM Cd at 9 weeks as compared to the control (Fig. 6.4). There was a large reduction in plant growth at 1000 µM Cd.

In Burke's Backyard, the Control leaf dry weight was not different from the Cd treatments (up to 500 µM Cd) after 3, 5, 7 and 9 weeks of exposure to Cd. At 1000 µM Cd, leaf dry weight was slightly reduced. This genotype showed moderate growth rate (Fig. 6.4).

In Grosse Lisse, the leaf dry weight was not different between the Cd treatments up to 500 µM Cd at 3, 5 and 7 weeks, but the dry weight decreased in 1000 µM Cd treatment. The highest leaf dry weight was found at week 9. This genotype had the slowest growth rate compared to all the genotypes and it accumulated 1 g leaf weight per plant except in week 9(Fig. 6.4).

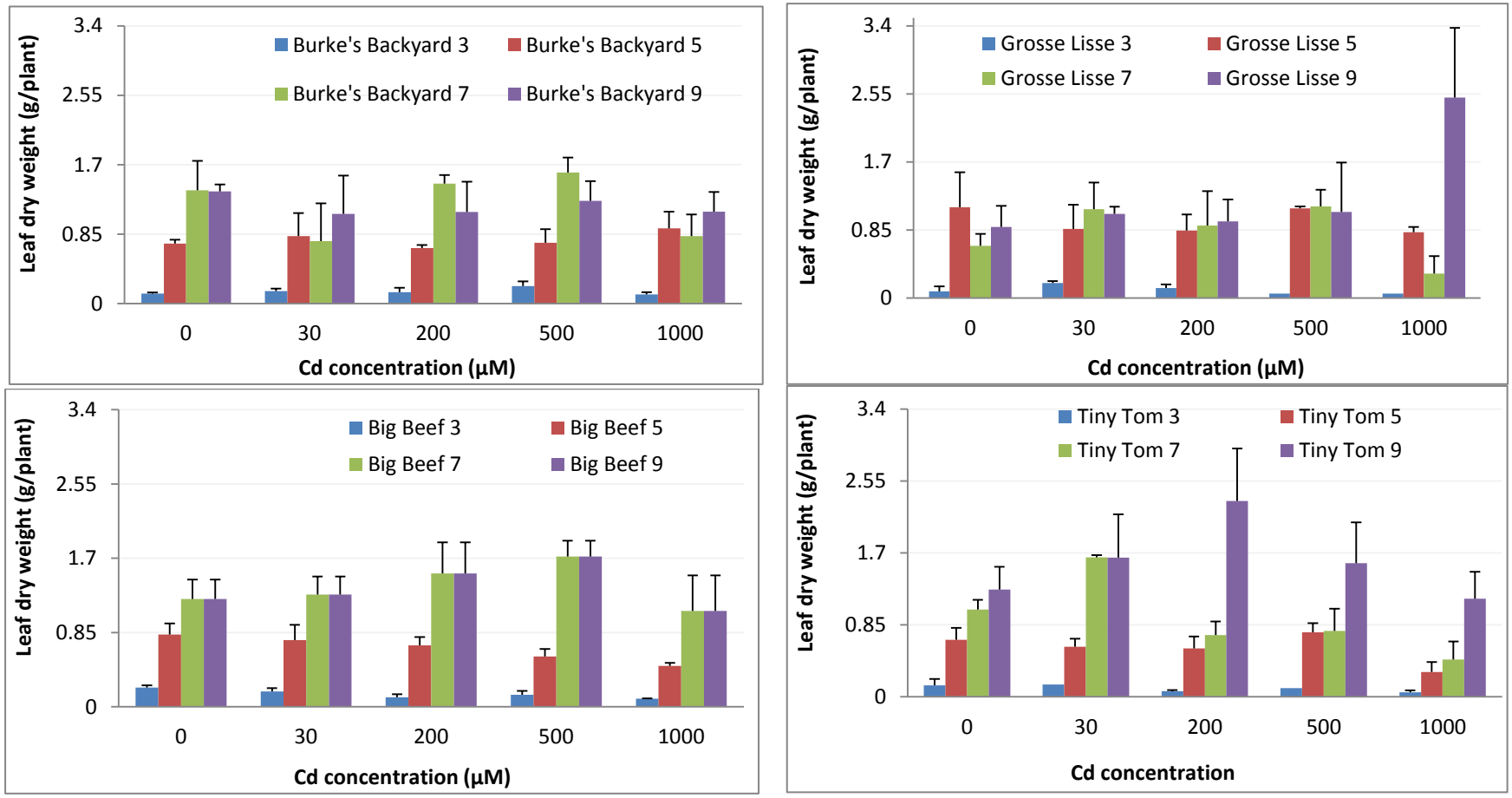


Figure 6.4 Leaf dry weight of 4 genotypes of tomato after 3, 5, 7 and 9 weeks of exposing to Cd in sand culture. Bars represent SE (n=3).

6.3.5 Effects of cadmium on stem dry weight

In genotype Big Beef, the stem dry weight increased with time in the control and 30 µM Cd, but its growth varied in other Cd treatments. Similar trends were noted for the other genotypes except in Big Beef, stem weight changed marginally with time at 1000 µM Cd (Fig. 6.5).

In Tiny Tom, the stem dry weight was markedly affected at 1000 µM Cd in weeks 3, 5, 7 and 9, and the trends varied for weekly harvests (Fig. 6.5). In Burke's Backyard, stem dry weight was not different between most Cd treatments after 3, 5 and 9 weeks of exposure to Cd (Fig. 6.5). The stem dry weight in Grosse Lisse was not different between the Cd treatments after 3 and 5 weeks. In any given treatment, large variation in stem weight was noted. This was because the seedlings that were harvested at each time varied in their shoots (Fig. 6.5).

The total biomass accumulation in each of the 5 treatments over 4 harvests are shown in Figure 6.5. It can be noted that most genotypes showed reduced growth at 1000 µM Cd (except Grosse Lisse). In this genotype, total biomass slightly increased at higher Cd levels. The proportion of leaf and stem however did not change either with the time or with the Cd (Fig. 6.5) except in Grosse Lisse at 1000 µM Cd and Tiny Tom at 200 µM Cd where the leaf weight was much higher than stem weight (Fig.6.6).

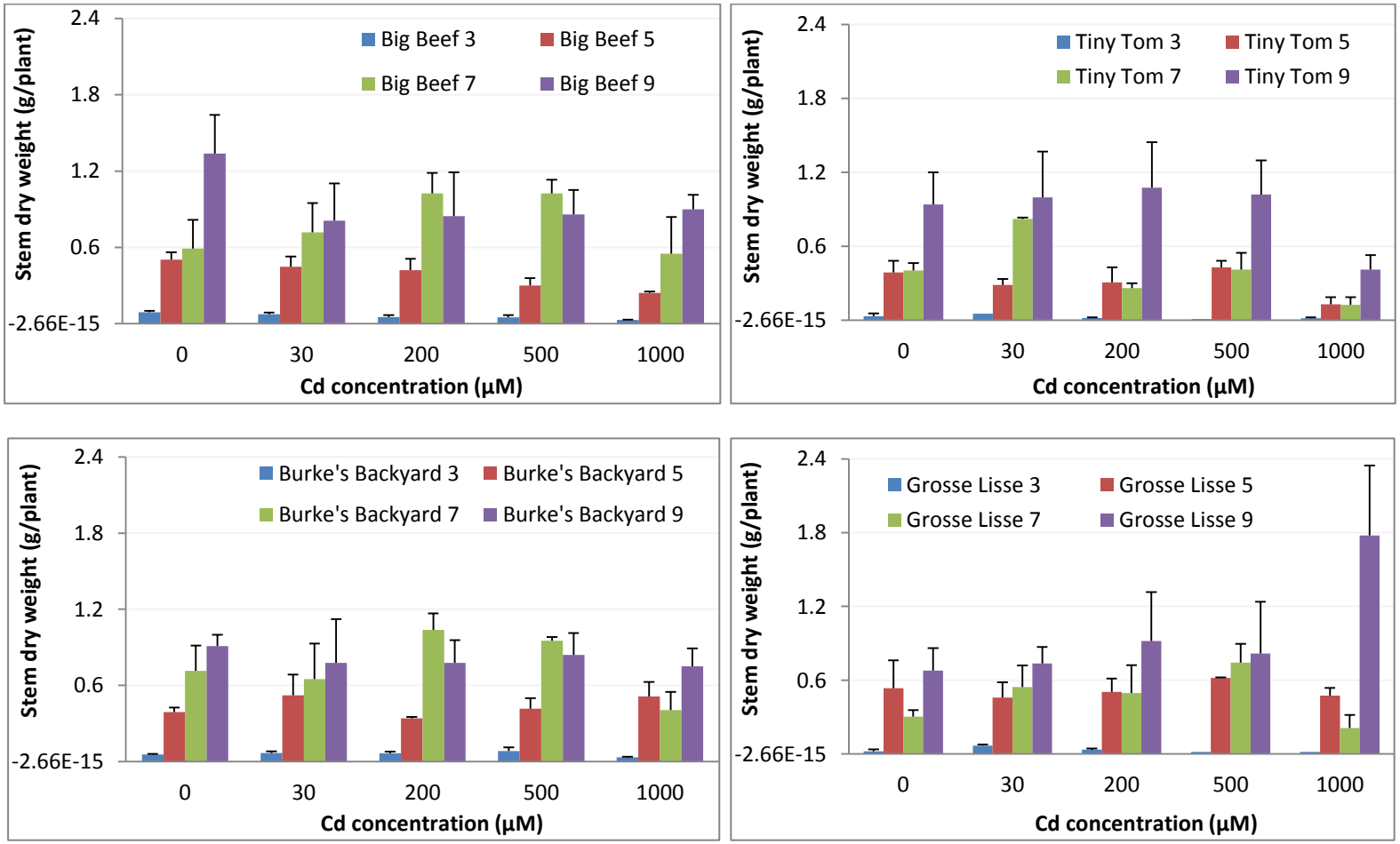


Figure 6.5 Stem dry weight (g) of 4 genotypes after 3, 5, 7 and 9 weeks of exposing to Cd. Bars represent SE (n=3).

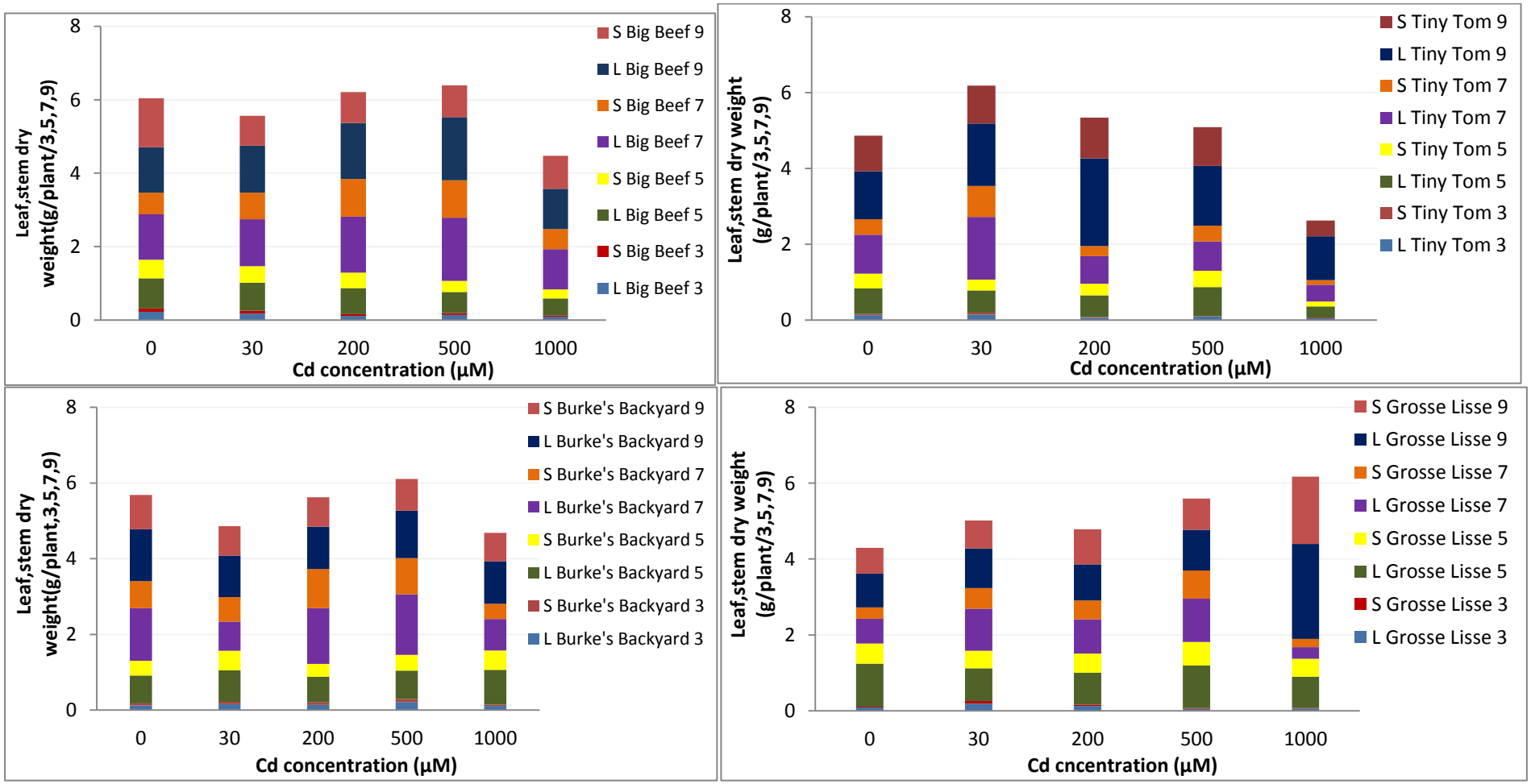


Figure 6.6 Leaf and stem dry weight of 4 genotypes of tomato at different Cd levels after 3, 5, 7 and 9 weeks of exposure.

6.3.6 Toxicity symptoms

Black spots were found on the leaves of Big Beef treated with 500 and 1000 µM Cd after seven weeks (Plate 6.2). The genotype Tiny Tom also had the black spots at 200 µM Cd after 7 weeks. No toxicity symptoms were found in Burke's Backyard and Grosse Lisse at all Cd levels even after seven weeks of growth. However, the older leaves of all genotypes changed to a purple colour at all Cd levels, viz. 30, 200, 500 and 1000 µM after 9 weeks, and the leaves of control also showed similar symptoms (Plate 6.2) .

Plate 6.2 Foliar symptoms of four tomato genotypes (left to right) Top: Big Beef, Tiny Tom; Bottom: Burke's Backyard and Grosse Lisse each treated with Cd; A1, B1, C1 & D1=control, A2, B2, C2 & D2=30 µM, A3, B3, C3 & D3=200 µM, A4, B4, C4 & D4=500 and A5, B5, C5 & D5=1000 µM Cd photos were taken after seven weeks of exposure to cd.

6.3.7 Leaf cadmium concentration

At 3 weeks, the highest Cd concentration (141µg/g) was found in Big Beef and the lowest (30 µg/g) in Tiny Tom at 1000 µM Cd (Fig. 6.7). The Cd concentration was not different amongst 4 genotypes at low Cd levels (30 and 200 µM Cd) but showed the highest variation at 1000 µM Cd.

The leaf Cd concentrations increased in all genotypes as the substrate Cd levels increased. At 3 weeks Cd concentration was 50µg/g, which increased up to 300µg/g at week 5 and 450µg/g in week 7. In week 9, the mean Cd concentration was 150µg/g. The latter is unusual. This may be due to inclusion of only recently produced leaves in the analysis as the older leaves had senesced (and were not included in the analysis) due to high temperature in the glasshouse (Fig. 6.7). This could also be due to harvesting soon after watering, in which case, the media Cd concentration would have been diluted.

The genotypes showed some difference in their leaf Cd accumulation. At week 3, Big Beef had higher Cd than other genotypes. At week 5, Grosse Lisse and Burke's Backyard had higher Cd at most Cd levels, with similar trends at week 7. Amongst the 4 genotypes Tiny Tom had lowest levels of Cd in its leaves at most concentrations. Overall, there was no clear trend between the genotypes in their leaf Cd accumulation in different Cd treatments and at different times of harvest, except in Tiny Tom which appears to accumulate lower levels of Cd at high (1000 µM Cd) Cd concentrations in week 5.

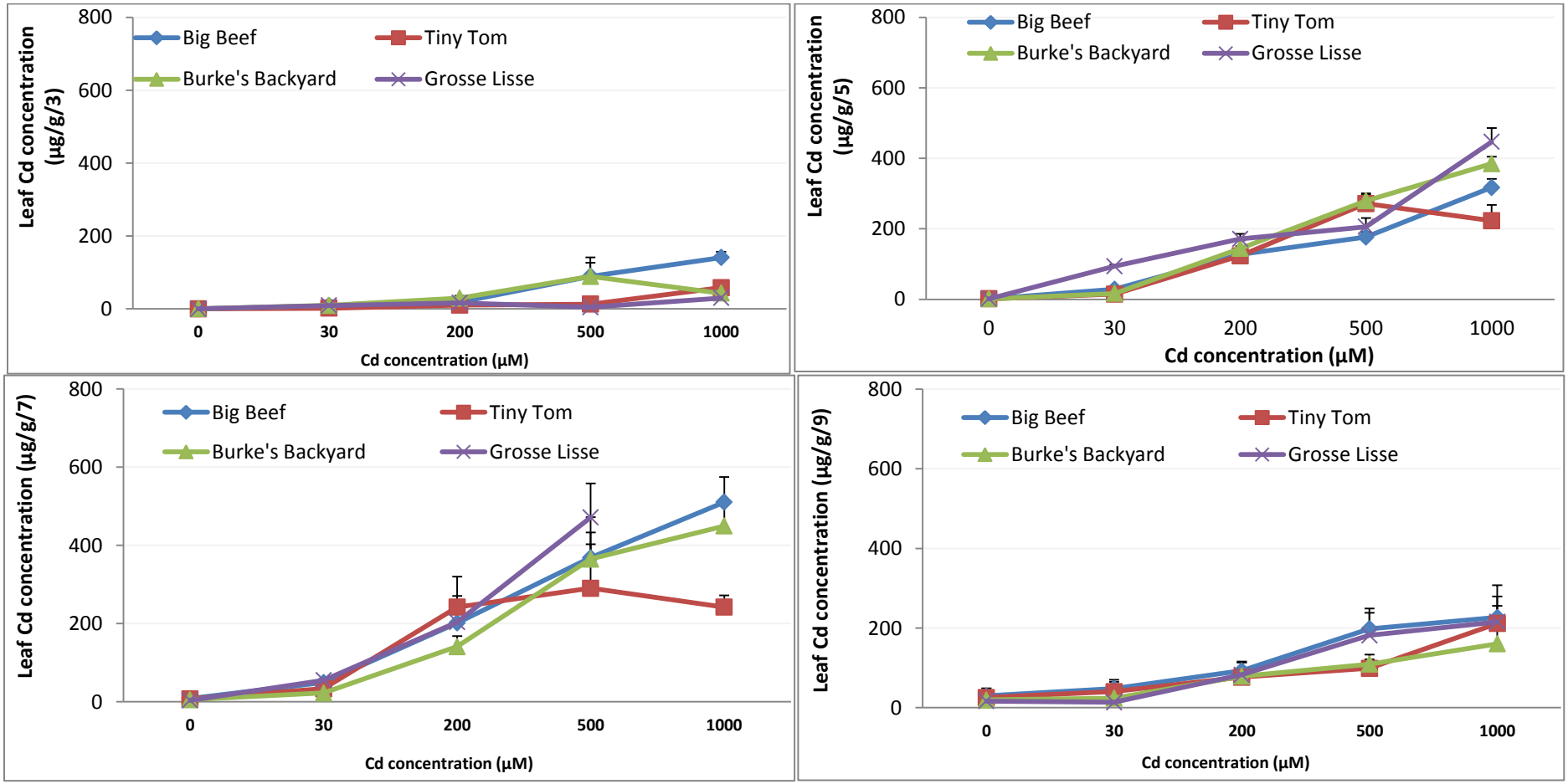


Figure 6.7 Leaf Cd concentrations (µg/g) in 4 genotypes at five levels of Cd (0, 30,200,500, 1000 µM) after 3, 5, 7 and 9 weeks of exposure to Cd. Bars represent SE (n=3).

6.3.8 Leaf Biological Absorption Coefficient

The highest leaf BAC value of 13 was found in the 3 genotypes at 30 µM Cd after 3 weeks of exposure to Cd. The lowest leaf BAC value of 1 was found in Tiny Tom at 200 µM Cd and in Grosse Lisse at 500 µM Cd. The Big Beef had higher BAC than Tiny Tom and Grosse Lisse at 200, 500 and 1000 µM Cd (Fig. 6.8).

At 5 weeks, the highest leaf BAC value (40) was found in Big Beef at 30 µM Cd, followed by Grosse Lisse was (36) at 200 µM Cd. The lowest leaf BAC value was found in Tiny Tom at 1000 µM Cd (Fig. 6.8).

At 7 weeks, the leaf BAC value was higher in Grosse Lisse (77) and Big Beef (69) at low Cd 30 µM Cd but at high Cd concentration (1000 µM) BAC reduced markedly in the 3 genotypes Tiny Tom (1), Burke's Backyard (2) and Grosse Lisse (2) (Fig. 6.8).

At 9 weeks, the highest leaf BAC value (50) was found in Big Beef and Tiny Tom at 30 µM Cd. The lowest leaf BAC value 7 was found in all 4 genotypes at 1000 µM Cd (Fig. 6.8). Amongst the 4 harvests, the BAC was high at week 5 and it was very low at week 9. Amongst all the Cd treatments, the BAC was high at lower medium levels of Cd levels than at higher Cd treatments.

Amongst the 4 genotypes, Big Beef had high BAC value especially at low Cd concentrations. At high Cd concentration its BAC was the similar to those recorded for other genotypes. Tiny Tom had low BAC value and the BAC value decreased with an increase in Cd concentration.

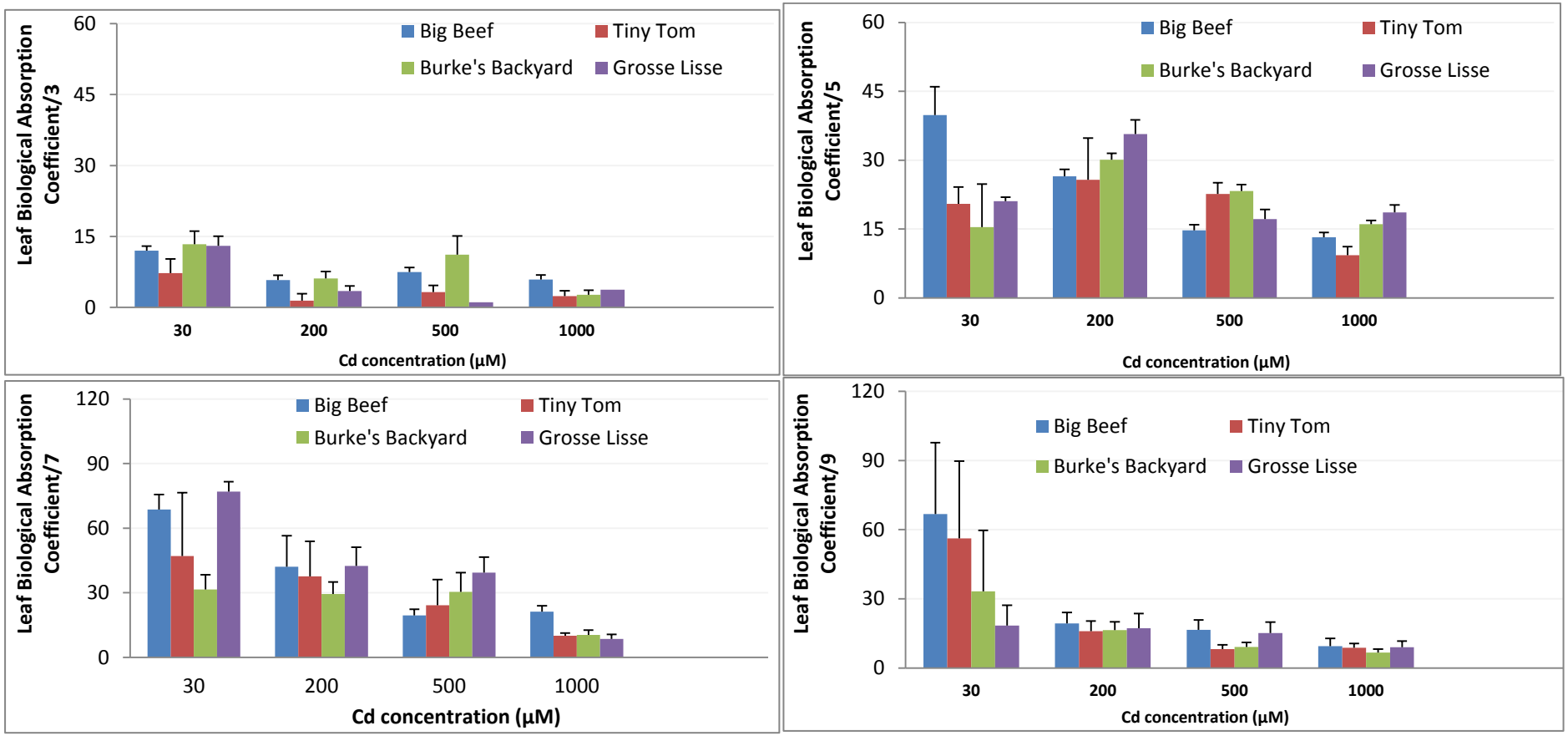


Figure 6.8 Leaf Biological Absorption Coefficient after 3, 5, 7 and 9 weeks of exposure to Cd.
Bars represent SE (n=3).

6.3.9 Cadmium uptake into leaves of four genotypes of tomato

At 3 weeks, the Cd uptake was low and there were no significant differences between the 4 genotypes at 30, 200 and 1000 µM Cd (Fig. 6.9).

At 5 weeks, the leaf Cd uptake varied in Big Beef and Grosse Lisse but mostly low in Tiny Tom at 1000 µM Cd. The highest Cd uptake was found in Grosse Lisse (373 µg/plant) and Burke's Backyard (363 µg/plant) and the lowest in Tiny Tom (74 µg/plant) at 1000 µM Cd (Fig. 6.9).

At 7 weeks, the leaf Cd uptake increased in 3 genotypes at 500 µM Cd compared to Tiny Tom. The highest Cd uptake 578 µg/plant was observed in Big Beef and the lowest (146 µg/plant) was in Tiny Tom at 500 µM Cd. There was no significant difference between the four genotypes at 30 and 200 µM Cd (Fig. 6.9).

At 9 weeks, the leaf Cd uptake increased in Grosse Lisse at 1000 µM Cd compared to other 3 genotypes. The genotypes Big Beef, Tiny Tom and Burke's Backyard were not different at 1000 µM Cd. The highest Cd uptake (436 µg/plant) was found in Grosse Lisse and the lowest 173 µg/plant was Burke's Backyard. There was no significant difference between the four genotypes at 30 and 500 µM Cd (Fig. 6.9).

The leaf Cd uptake increased with time in all genotypes in weeks 3, 5 and 7 except in Tiny Tom which showed reduced uptake at 1000 µM Cd.

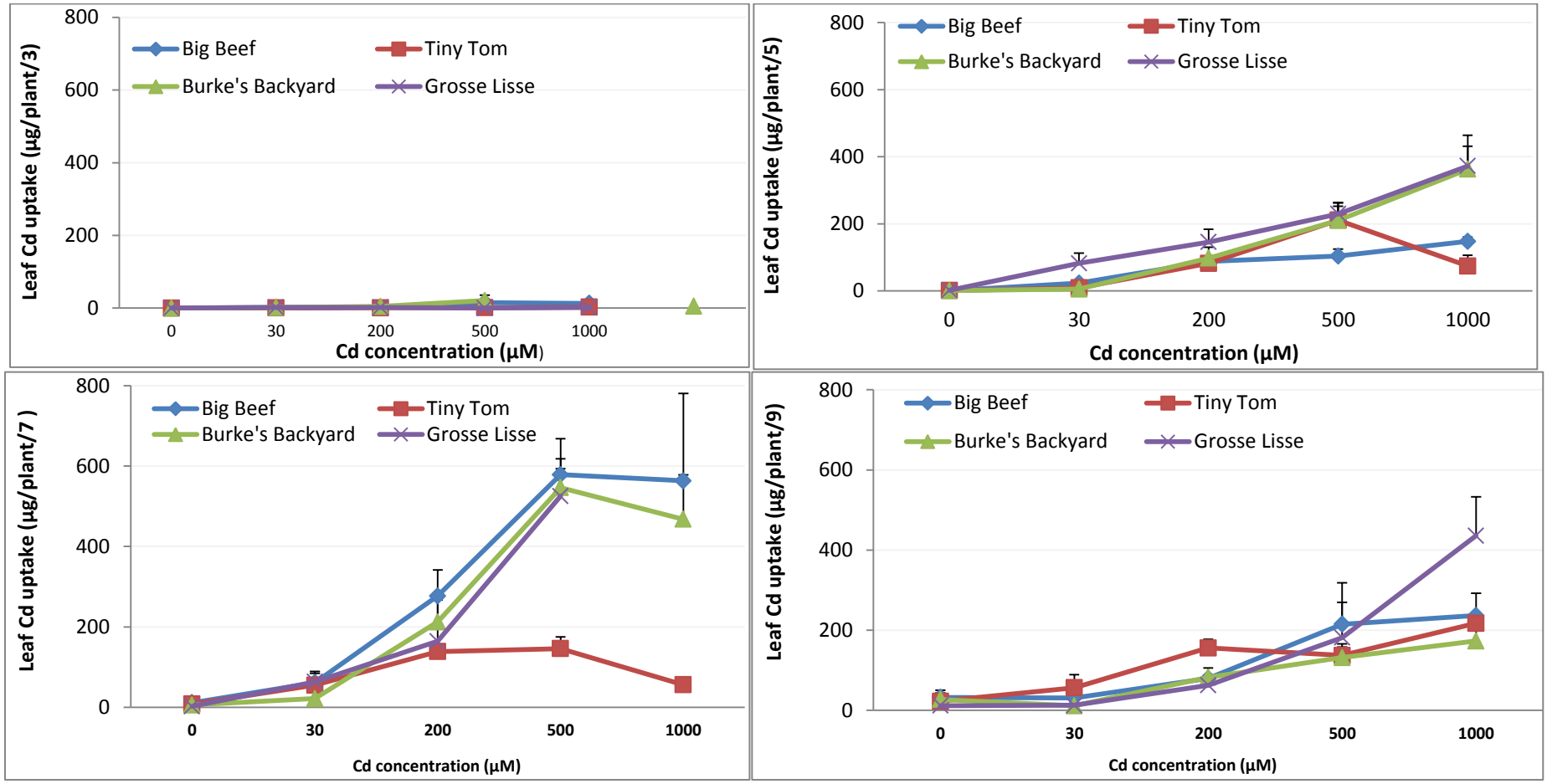


Figure 6.9 Leaf cadmium uptakes (µg/plant) in four genotypes after 3, 5, 7 and 9 weeks.
Bars represent SE (n=3).

6.3.10 Stem cadmium concentration and uptake

In week 7, the stem Cd concentration increased in Big Beef and Burke's Backyard at 1000 µM Cd but slightly decreased in Grosse Lisse at 1000 µM Cd. The highest Cd concentration (210 µg/g) was observed in Big Beef and the lowest (130 µg/g) in Tiny Tom at 1000 µM Cd. No significant differences were noted between the genotypes at 30 and 200 µM Cd (Table 6.1).

In week 7, the stem Cd uptake increased in genotype Big Beef, Burke's Backyard and Grosse Lisse but did not change markedly in Tiny Tom, as the substrate Cd concentrations increased. The highest Cd uptake (148 µg/plant) was found in Big Beef and the lowest (17 µg/plant) in Tiny Tom at 1000 µM Cd. There was no significant difference between the four genotypes at 30 µM Cd (Table 6.1).

The leaf Cd concentration was higher than stem Cd concentration in 4 genotypes at week 7 (Fig. 6.11).

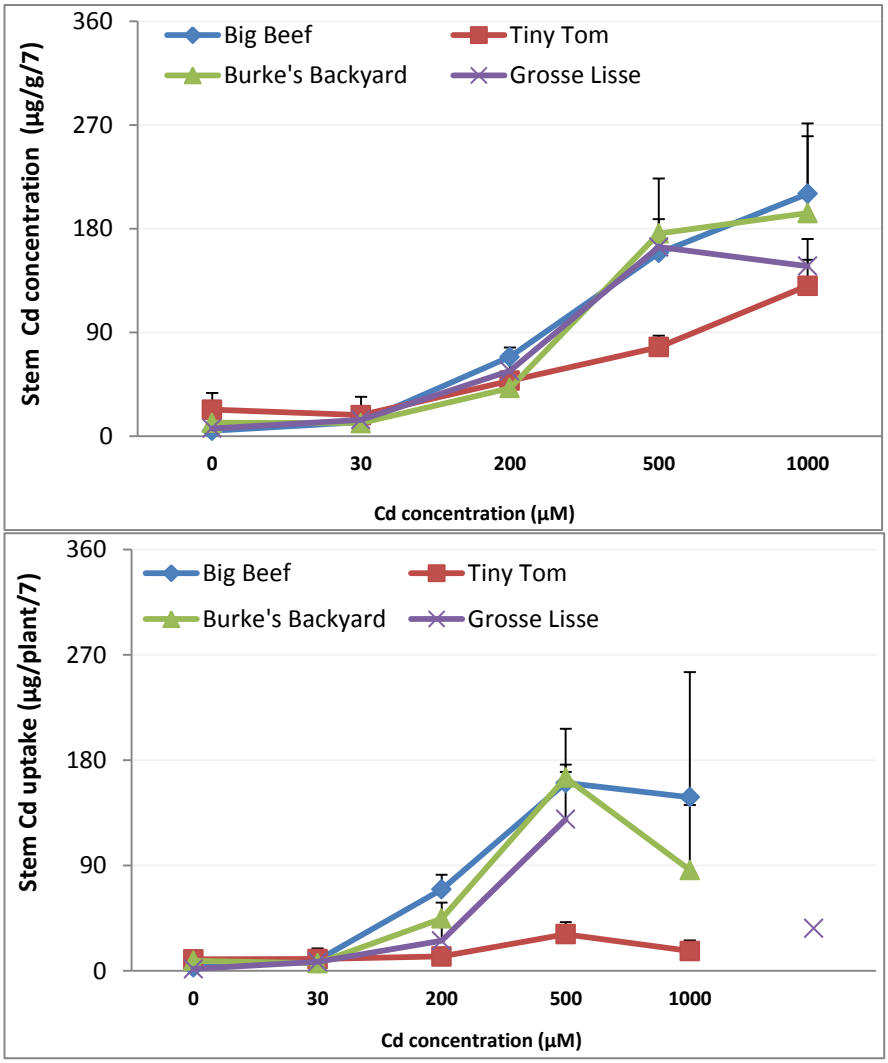


Figure 6.10 Stem cadmium concentration (µg/g) and uptake (µg/plant) in 4 genotypes after 7 weeks. Bars represent SE (n=3).

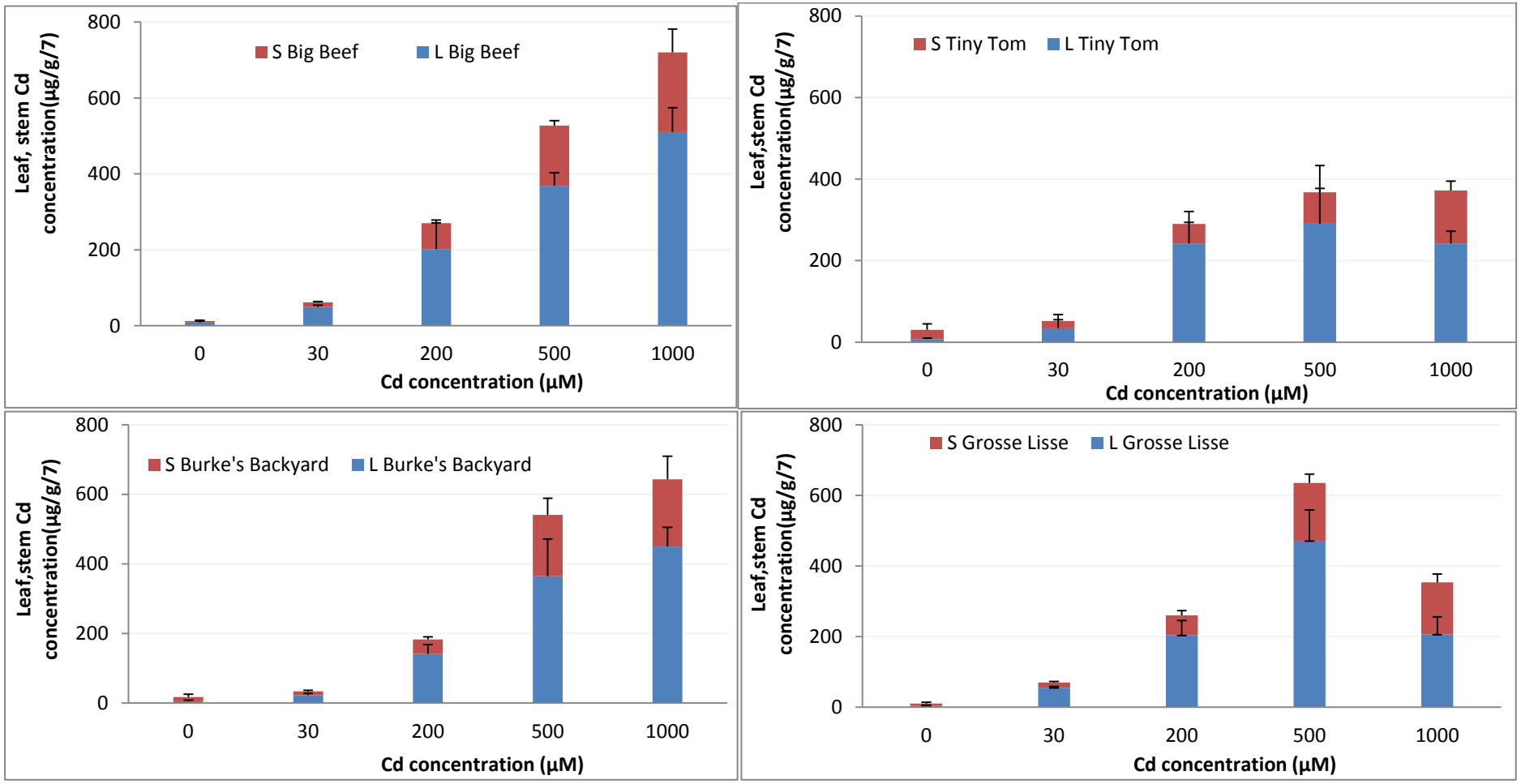


Figure 6.11 Leaf and stem Cd concentration in 4 genotypes after 7 weeks of exposure to Cd.
Bars represent SE (n=3).

6.3.11 Shoot cadmium concentration and uptake in four genotypes

At week 7, the shoot cadmium concentration increased in Big Beef, Burke's Backyard and Grosse Lisse but it plateau at 500 µM Cd in Tiny Tom. The highest shoot Cd concentration 421 µg/g was recorded in Big Beef and the lowest 184 µg/g in Tiny Tom at 1000 µM Cd. There was no significant difference between the four genotypes at 30 and 200 µM Cd (Fig. 6.12).

At week 7, the shoot Cd uptake increased in genotypes Big Beef, Grosse Lisse and Burke's Backyard but the increase was marginal for Tiny Tom up to 500 µM Cd (Fig. 6.12). The highest shoot Cd uptake 712 µg/plant was observed in Big Beef but the lowest 54 µg/plant was in Tiny Tom at 1000 µM Cd. The shoot cadmium uptake was not markedly different between the four genotypes at 30 µM Cd.

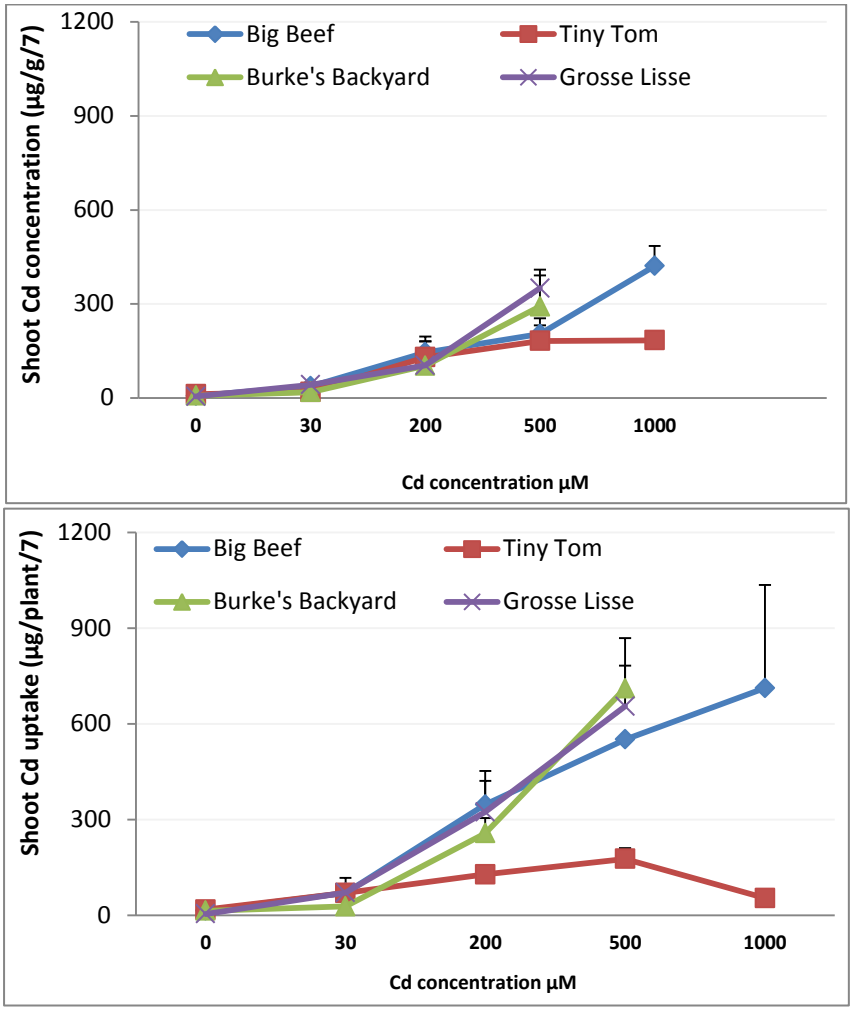


Figure 6.12 Shoot Cd concentrations (µg/g) and shoot Cd uptake (µg/plant) in 4 genotypes after 7 weeks of exposure to Cd.

6.3.12 Shoot Biological Absorption Coefficient

The high shoot BAC of 232 and 244 was found in Big Beef and Burke's Backyard at 1000 µM Cd after 7 weeks of exposure to Cd. The lowest shoot BAC value 43 was found in Tiny Tom and Burke's Backyard at 30 µM Cd. Overall, Tiny Tom had the lowest BAC compared to other genotypes at all levels of Cd (Fig. 6.13).

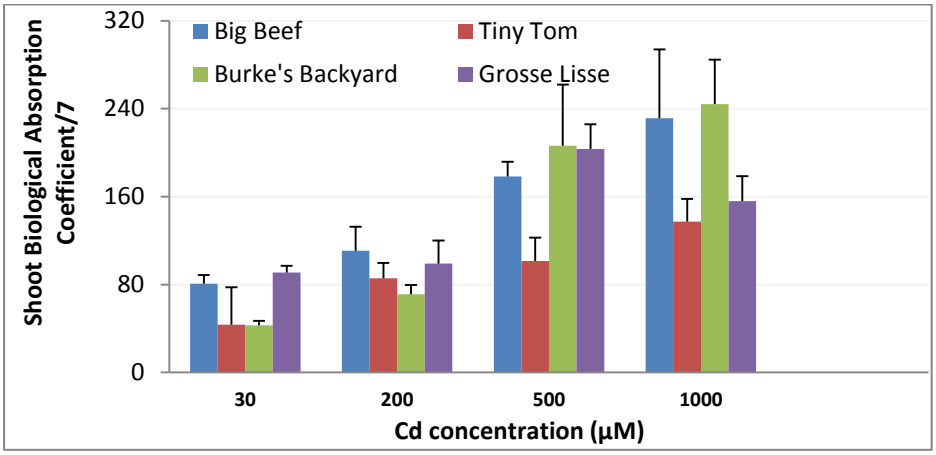


Figure 6.13 Shoot Biological Absorption Coefficient of 4 genotypes of tomato after 7 weeks of exposure to Cd

6.3.13 Comparison between tissue culture and sand culture for Cd accumulation

Based on plant tissue culture and glasshouse sand culture experiment, selected 4 genotypes were compared with their leaf Cd concentration after 5 weeks of exposure to Cd. The results indicated that the relative response of genotypes in tissue culture experiment was similar to that observed in sand culture. However, the leaf Cd concentrations were up to 4 times higher in *in vitro* cultured plants than in the plants grown in glasshouse in sand culture. This discrepancy is possibly due to increased availability of Cd in the tissue culture media (Fig. 6.14).

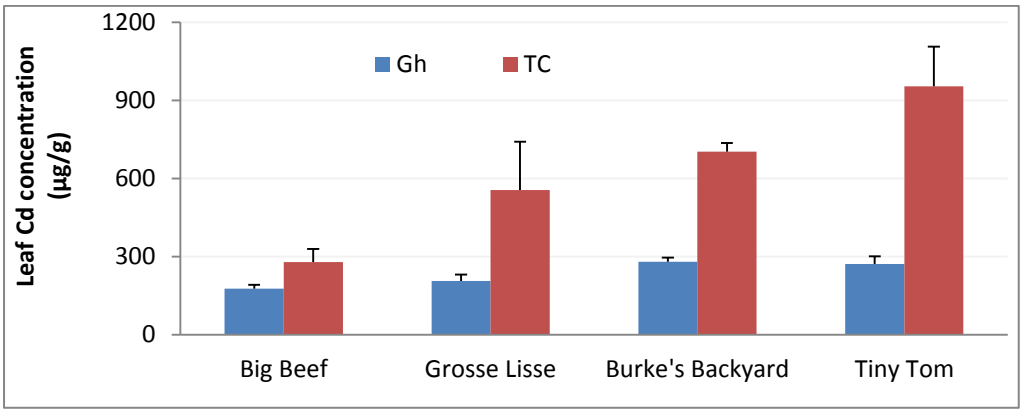


Figure 6.14 Compare with tissue culture (TC) and glasshouse sand culture (Gh) of Cd concentration.

Table 6.1 Values are *P* values from ANOVA for 4 genotypes

	df	G%	Ht	9LDW	9SDW	7LDW	7SDW	7L Cd uptake	7L Cd con	7S Cduptake	7S Cdconc	7 Shoot conc	7 Shoot uptake
Cultivar	3	0.652 NS	<.001***	0.107 NS	0.637 NS	0.002**	<.001***	0.040*	0.805	0.023*	0.192	0.13	0.010**
treatment	4	0.666 NS	0.394 NS	0.712 NS	0.911 NS	0.016*	<.001***	<.001***	<.001	<.001***	<.001	<.001***	<.001***
Cultivarv*treatment	12	0.650 NS	0.228 NS	0.027 *	0.048 *	0.561NS	0.561NS	0.291	0.564	0.342	0.413	0.047*	0.218

	df	No. of Leaves	9LCdcon	9Luptake	3LDW	3SDW	3LCdcon	3Luptake	5LDW	5LCdconc	5Luptake	5SDW
Cultivar	3	0.604	0.302 NS	0.960 NS	<.001***	0.002**	0.005**	0.031*	0.009**	<.001***	<.001***	0.010*
treatment	4	0.268	<.001***	<.001***	0.037 *	0.002**	<.001***	0.010**	0.437 NS	<.001***	<.001***	0.447 NS
Cultivarv*treatment	12	0.285	0.552 NS	0.256 NS	0.056*	0.318 NS	0.018*	0.179 NS	0.805 NS	<.001***	0.002**	0.470 NS

Note: 3, 5, 7, 9 denotes weeks after exposure to Cd. DW=dry weight, L= leaf, Ht= height, S= stem, con= concentration.

6.4 Discussion

The Cd tolerance and hyperaccumulation was investigated using 4 genotypes of tomato. The present experiment showed that the four genotypes of tomato were tolerant to high Cd concentrations. The highest seed germination (100%) was found in Grosse Lisse and Tiny Tom at 1000 µM Cd. Kuriakose and Prasad (2008) reported that the seed germination decreased in *Sorghum bicolour* treated with 3000 µM Cd as compared to control. Seed germination in pea was not affected by cadmium at 500 µM but it decreased seed germination at 1000 µM Cd compared to control (Chugh and Sawhney 1996). The seed germination of oil seed rape (*Brassica napus*) was sensitive to 200 and 400 µM Cd (Meng *et al*. 2009).

In the present study, plant height was not different between high Cd levels. Yang *et al.* (2004) reported *Sedum alfredii* plant shoot height decreased at 800 µM Cd. The plant height of two tomato cultivars Hezuo 903 and Jiang-shu 14 decreased at 10 µM Cd but no significant differences were noted at 0.1 µM (Dong *et al.* 2005).

In the present experiment, the leaf numbers were not different in Big Beef, Burke's Backyard and Grosse Lisse at high Cd concentration. The leaf numbers of tomato cultivars decreased at 10 µM Cd but no significant difference were found at 0.1 µM (Dong *et al.* 2005). Lefevre *et al.* (2005) reported reduction in the number of leaves in *Zygophyllum fabago* at 10 µM Cd compared to control.

The present experiment, leaf dry weight decreased in Big Beef at 1000 µM Cd after 5 weeks and not at week 3, 7 and 9. Dong *et al.* (2005) have reported a reduction in leaf dry weight in tomato at 10 µM Cd when compared to control. The shoot dry weight of *Sedum alfredii* decreased at 800 µM Cd but increased at 100 µM Cd compared to control (Yang *et al*. 2004). The leaf dry weight of Arum decreased at 50

µM Cd and the shoot dry weight of Radish and leaf dry weight of Water spinach reduced at 10 µM Cd (Kashem *et al.* 2008). Lopez-Millan *et al*. (2009) have also reported reduction in fresh weight and dry weight of leaf and stem in tomato at 100 µM Cd compared to control.

In the present study, stem dry weight decreased in Tiny Tom at high Cd level after 5 weeks. There was no difference between Cd treatments in Big Beef and Burke's Backyard after 3, 5, 7 and 9 weeks. Lopez- Millan *et al*. (2009) reported that the stem dry weight decreased in tomato at 100 µm Cd when compared to control. Zorrig *et al*. (2010) found that the shoot weight decreased in lettuce treated with 50 µM Cd compared to control. The stem dry weight decreased in arum at 50 µM Cd but not at 10 µM Cd as compared to control. In radish and water spinach stem dry weight decreased at 10 µM Cd compared to control (Kashem *et al*. 2008).

The present experiment, after seven weeks of Cd exposure, black spots were observed in Big Beef treated with 500 and 1000 µM Cd and in Tiny Tom at 200 µM Cd respectively. The older leaves changed to a purple colour in all four genotypes at all Cd levels (30, 200, 500 and 1000 µM) after 9 weeks. Leaf necrosis and high plant toxicity symptoms were observed in tomato cultivar Hezuo 903 and Jiang shu 14 treated with a range of Cd from 1 µM to 10 µM (Dong *et al*. 2005). In hydroponics culture, no symptoms were found in *Brassica jounce* treated with 0.24 µM Cd (Ishikawa *et al.* 2006). Chlorosis and white spots were found in young leaves of radish and water spinach at 1.5- 10 µM Cd (Kashem *et al.* 2008).

Shoot Biological Absorption Coefficient (BAC) value was higher than 1 in 4 genotypes at all Cd levels after 3, 5, 7 and 9 weeks of exposure. Therefore, these 4

genotypes can be used for phytoextraction. Vamerali *et al.* (2010) mentioned that plants with high BAC value greater than 1 are suitable for phytoextraction.

Malik *et al*. (2010) reported the highest BAC value of Pb was found in cultivar *Brachiaria reptans* (14) and *Cynodon dactylon* (4.6). The BAC value decreased in cultivar *Brassica juncea* and *Grevillea exul* at 1000 mg/kg Ni (Rabier *et al*. 2007).

In the present study, the leaf Cd concentration was higher in all genotypes at 1000 µM Cd after 5 and 7 weeks but lower at 3 and 9 weeks of exposure to Cd. The leaf Cd uptake increased in 3 genotypes Big Beef, Burke's backyard and Grosse Lisse in high Cd levels at week 5 and 7 but reduced at week 3 and 9. The highest stem Cd concentration and Cd uptake was observed in Big Beef at 1000 µM Cd after 7 weeks of exposure.

Zorrig *et al*. (2010) reported that the cadmium contents sharply increased in three lettuce cultivars treated with 50 µM Cd compared to control. The highest Cd accumulation was observed in the shoots of rice and sugar beet compared to other cultivars maize and *Brassica juncea* that were treated with 60 µM Cd (Ishikawa *et al*. 2006). The Cd accumulation in tomato shoots at 500 µM Cd was 50 times more concentrated than that at 10 µM Cd after 20 days of Cd exposure (Cho and Park 1999). The cadmium concentrations increased in the leaves and stems of *Sedum alfredii* treated with 400 µM Cd compared to control. The leaf cadmium uptake of shoots and roots linearly increased in *Sedum alfredii* at 400 µM but decreased at 800 µM (Yang *et al*. 2004). Shoot Cd uptake increased in pea at 30 µM Cd compared to 1and 3 µM Cd (Lima *et al*. 2006).

In conclusion, the results indicated that the cadmium toxicity did not affect seed germination of four tomato genotypes. The plant height of Grosse Lisse was tolerant

to high Cd concentration (1000 µM) but Tiny Tom was sensitive to Cd. The leaf dry weight was higher than stem dry weight after 3, 5, 7 and 9 weeks. The higher Cd concentration and uptake was found in Big Beef after 3 weeks and 5 weeks and in genotype Grosse Lisse after 5 weeks and 9 weeks. The genotypes Big Beef, Grosse Lisse and Burke's Backyard could be used for phytoremediation of cadmium polluted soil provided the initial maximum Cd concentration was around 500 µM.

These results indicated that the relative response of genotypes in tissue culture experiment was similar to that observed in sand culture. However, the leaf Cd concentrations was up to 4 times higher in *in vitro* cultured plants than those grown in the glasshouse in sand culture.

Chapter 7

Long term uptake and distribution of Cd in two genotypes of tomato

7.1 Introduction

Heavy metals in soils are derived from natural and human activities. Plants show various symptoms in response to metal uptake, changing their physiology and morphology when exposed to heavy metals (Sakalauskaite *et al.* 2006). According to FAO, human beings can tolerate 70-mg/day of Cd when they consume food and drinking water containing Cd (Cesur and Kartal 2007). Hyperphosphate rocks contain cadmium ranging from 0.1 to 11 mg/kg (Cesur and Kartal 2007). The use of sewage sludge from industry and human activities causes metal pollution in soils which range from <1 mg/kg to 100,000 mg/kg (Aydinalp and Marinova 2009). Industries produce polluted gas and metals from electrolysis. Cadmium contaminated environment may affect to human health due to smelting, the combustion of fuels, the production process of tyres and release of waste products by the industries (Williams and David 1973). In agriculture, the application of phosphate fertilizers, organic manure and pesticides can cause metal contamination in the soil environment (Chehregani *et al.* 2009). Edible plants grown in metal polluted soils have higher health hazards than those grown in unpolluted soils (Kachenko and Singh 2006).

Plant growth and quality are influenced by nutrient absorption and soil pH (Januskaitiene *et al.* 2004). Reductions in plant growth, cell proliferation and increases in membrane conductance are affected by cadmium toxicity (Januskaitiene 2004). Cadmium is highly mobile in the soil and hence it can contaminate the soil environment. Cadmium is easily accumulated in the edible parts of plants so when humans consume the contaminated plants they will be exposed to health risks (Li *et al.* 2010). Inhibition of photosynthesis and reduction of water and nutrient absorption are symptoms of phytotoxicity due to cadmium accumulation in plants (Haider *et al.* 2007). Different plants have different Cd uptake rates. Thus it would be useful to select cultivars with a low Cd uptake in order to reduce the risk of Cd toxicity to human beings (Vasiliadou and Dordas 2009). Metal contaminated soils and water can be remediated by ion exchange, electrostatic precipitation, reverse osmosis, evaporation and chemical reduction. However, this process is costly (Mangkoedihardjo and Surahmaida 2008). Phytoremediation is the use of plants capable of hyperaccumulating metals in the cleanup processes in a contaminated soil environment. Metal hyperaccumulator plants can take up more than 10,000µg/g for Zn, more than 1000µg/g for Ni and more than 100µg/g for Cd (Baker *et al*. 1994; Brown *et al*.1994). Phytoremediation is a cost effective and environmentally-friendly technique and this can be used by the environmentalists, farmers and researchers.

The aim of this research is to compare the uptake, accumulation and tolerance of two tomato genotypes that were grown in sand culture for 17 weeks. The specific objectives include:

1. Compare two tomato genotypes for their ability to accumulate cadmium in their leaves and stems over longer term (17 weeks).

2. Compare the two genotypes for their growth, chlorophyll contents and distribution of Cd in leaves and stems in response to addition of different concentrations of cadmium to their growth media.

7.2 Materials and Methods

7.2.1 Selection of genotypes

Seeds of two tomato genotypes, viz., Big Beef (produced by Yates) and Grosse Lisse (produced by Mr. Fothergill's) were selected for the study based on the previous experiment (see Chapter 5) and the seeds were purchased from Bunning's Warehouse, Rockhampton. The reasons for selecting these genotypes are:

1. Grosse Lisse: low Cd accumulator (see in Chapter 5)
2. Big Beef: high Cd accumulator

7.2.2 Pot culture

Eighteen large plastic polyethylene pots (20 x 15.8 x 19.2 cm) were used in this study and were set up in a glasshouse as shown in Plate 7.1. Saucers were placed under the pots to collect drained solution, allowing the drained solution to be reabsorbed by the plant.

Plate 7.1 Pot cultures of two genotypes in a glasshouse.

Washed river sand obtained from Rockhampton Mini Loads and was dried for two days at 60˚C. Dried sand (7 kg) was filled into each plastic pot. The pots were placed on the saucer to ensure that the excess water was retained and not drained. All pots were placed on a glasshouse bench (Plate 7.1).Cadmium stock solution (100 mM) was prepared as described in Chapter 3.6. Ten seeds of each genotype were sown in each pot and were watered with demineralised water until seed germination. After 2 weeks, the seedlings were thinned to retain six plants per pot. Three treatments of Cd were imposed (0, 100 and 500 µM Cd) and the treatments were replicated three times. Hydroponics solution was prepared as described in Chapter 3.7. When the seedlings grew to 3 cm, hydroponics nutrient solution with or without Cd were poured into pots at 200 ml/pot for the first week and 200 ml/pot for the second week. The glasshouse was maintained at a temperature of 20-28°C using an evaporative cooler.

7.2.3 Growth parameters

Seed germination, plant height, leaf number per plant, fruit number per plant, fresh and dry weight of fruit, leaf and stem dry weight, number of flower bunches, number of branches per plant, number of nodes, length of inter node and cadmium concentration and uptake in their shoots were determined after exposing the plants to cadmium. The chlorophyll content was measured using digital- chlorophyll-SPAD-502 Plus meter. The SPAD readings were recorded 9 weeks after exposing the plants to Cd. The stem diameter was measured by a 150 mm stainless steel digital calliper. Morphological symptoms of the plants in response to Cd treatments were recorded via photographing.

7.2.4 Tissue analysis for cadmium

The plants were harvested at 7 and 17 weeks after sowing. The shoots were separated into leaves and stems. The leaves were counted and dried in an oven at 72°C over 3 days, and the dry weights of the shoots were recorded. Plant samples were ground into a fine powder (<1.5 mm) using a Culatti hammer mill (MFC Mikro-Feinmuhle-Culatti). The powder (0.4 g) was placed in 10 ml plastic tube (PPTR) and 2 ml of nitric acid (HNO_3) was added along with a drop of hydrogen peroxide (H_2O_2). The plastic tubes were placed in the fume hood night over. On the following morning, 3 ml of Milli-Q water was added and then placed in a water bath (Grant Instruments, Cambridge Ltd) maintained at 70°C for 4 hours. After digestion, the volume of tube was made up to 10 ml using Milli-Q water. The tube was centrifuged at 15,000 gfor 15 minutes. The Cd concentrations of the supernatant were determined using AAS spectrophotometer (see Chapter 3.8).

7.2.5 Biological Absorption Coefficient (BAC)

Biological Absorption Coefficient (BAC) was calculated according to Malik *et al*. (2010) as the ratio of cadmium concentrations in shoots to that in the soil as:

$$\text{BAC uptake (µg/g/plant)} = \frac{\text{Metal concentration in shoots}}{\text{Metal concentrations in the growth media}}$$

7.2.6 Statistical analysis of data

Analysis of variance was performed using Genstat version 13 following testing the data for outliers, normality and homogeneity of error variances. Standard errors of means or least significant differences (lsd) were used for comparing means.

7.3 Results

7.3.1 Effects of cadmium on seed germination

There were no significant differences between the two genotypes in seed germination (Fig. 7.1). However, the seeds in 500 µM Cd treatment had slight reduction in germination percentage in Big Beef.

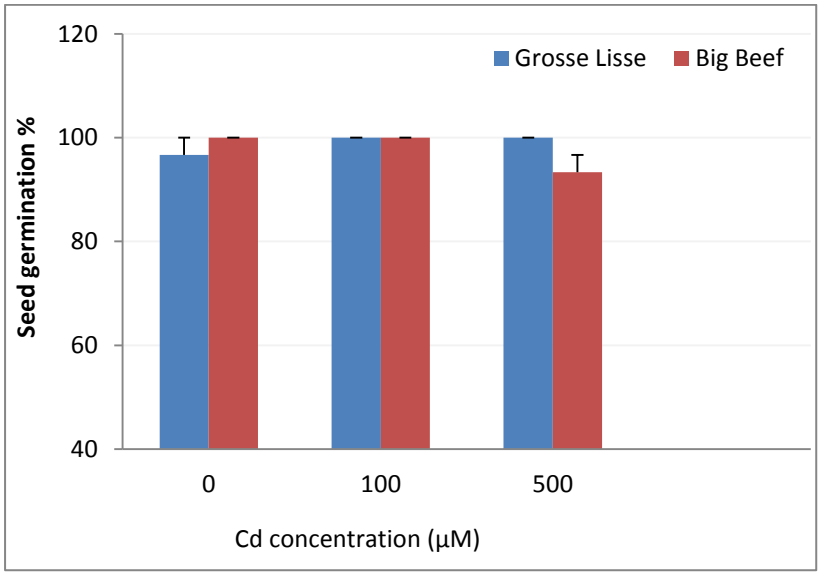


Figure 7.1 Seed germination percentage of 2 genotypes, Grosse Lisse and Big Beef

7.3.2 Effect of cadmium on plant height

The plant height was significantly (P<0.05) increased in Big Beef at 100 and 500 µM Cd when compared to the control, but had little effect on Grosse Lisse (Fig. 7.2).

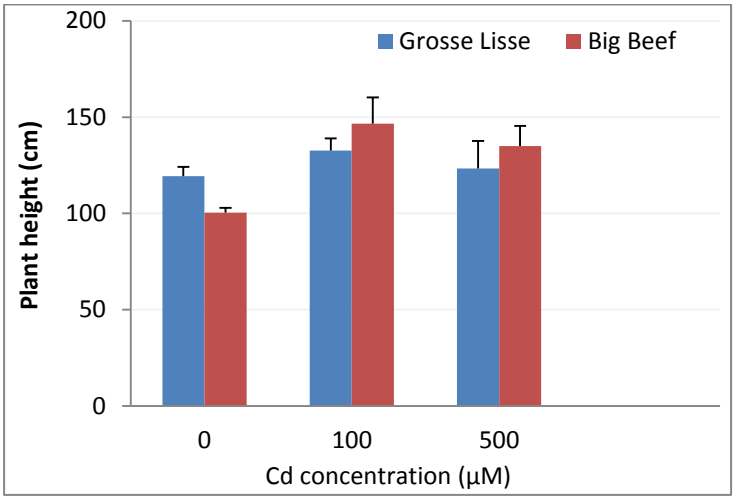


Figure 7.2 Shoot height of the 2 genotypes of tomato treated with or without Cd for 17 weeks after exposure to Cd.
Bars represent SE (n=3).

7.3.3 Effects of cadmium on leaf numbers, fruits and dry weight of fruit

The number of leaves decreased in Big Beef treated with 500 µM Cd as compared to the control (Fig. 7.3). Little differences occurred between Cd treatments in Grosse Lisse.

Both control and Cd treated plants produced very few fruits. No fruits were developed in Big Beef at 100 µM Cd and Grosse Lisse at 500 µM Cd but a few fruits were found in Grosse Lisse at 100 µM Cd and at 500 µM Cd in Big Beef (Fig. 7.3).

The control and treated plants did not produce sufficient fruits because of high temperature in the glasshouse during summer. However, amongst those produced fruit dry weight was not affected by Cd treatment (Fig. 7.3).

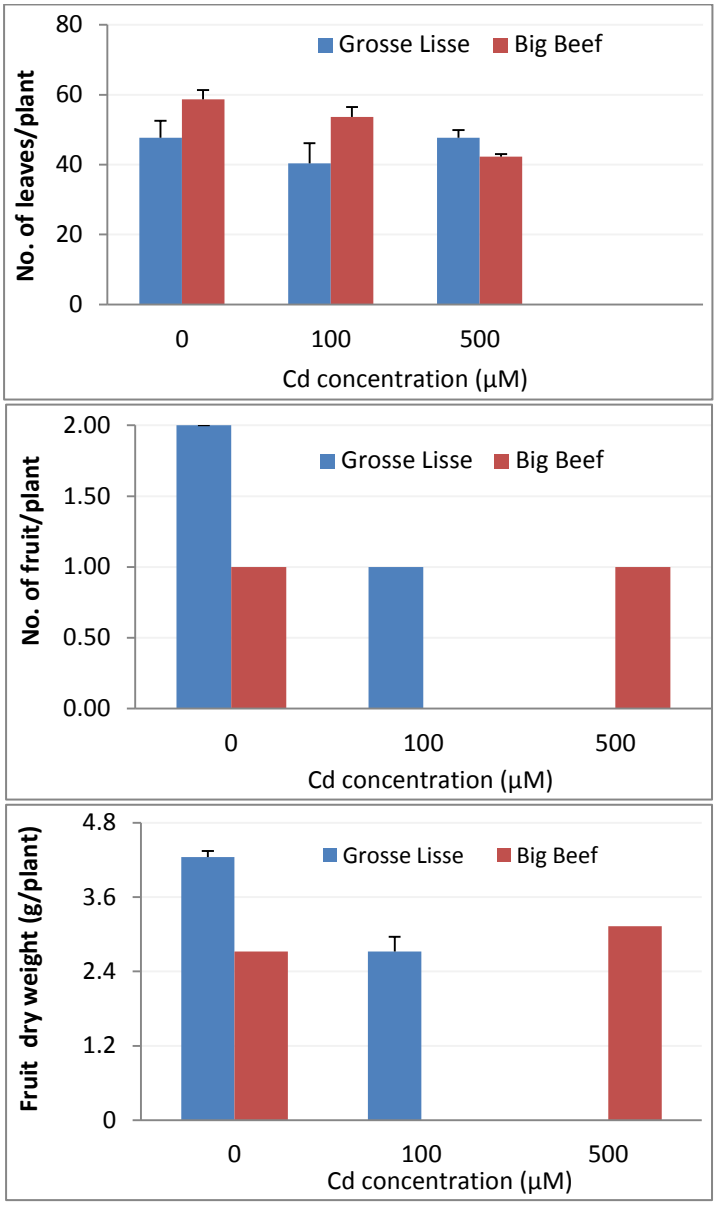


Figure 7.3 No. of leaves, fruits and dry weight of fruits in 2 genotypes of tomato after 17 weeks of sowing.
Bars represent SE (n=3).

7.3.4 Effect of cadmium on leaf and stem dry weight

At 7 weeks, there was no difference in leaf dry weight between the treatments in Grosse Lisse. However, leaf dry weight decreased in Big Beef at 500 µM Cd as compared to the control (Fig. 7.4).

At 17 weeks, there was no difference between the treatments (P<0.05) (Table 7.1) in Grosse Lisse. The leaf dry weight slightly increased in Big Beef at 100 and 500 µM

Cd as compared to the control (Fig. 7.4). The highest leaf dry weight was observed in both genotypes after 17 weeks when compared to 7 weeks.

In Grosse Lisse, stem dry weight was not affected by Cd treatment. However it declined significantly at 500 µM Cd in Big Beef at 7 weeks of harvest (Fig. 7.4).

At 17 weeks, the stem dry weight increased up to 8 times compared to that of 7 weeks (Fig. 7.4). The stem weight of Big Beef increased at 100 µM Cd, when compared to control, and no difference occured with the Cd treatments in Grosse Lisse.

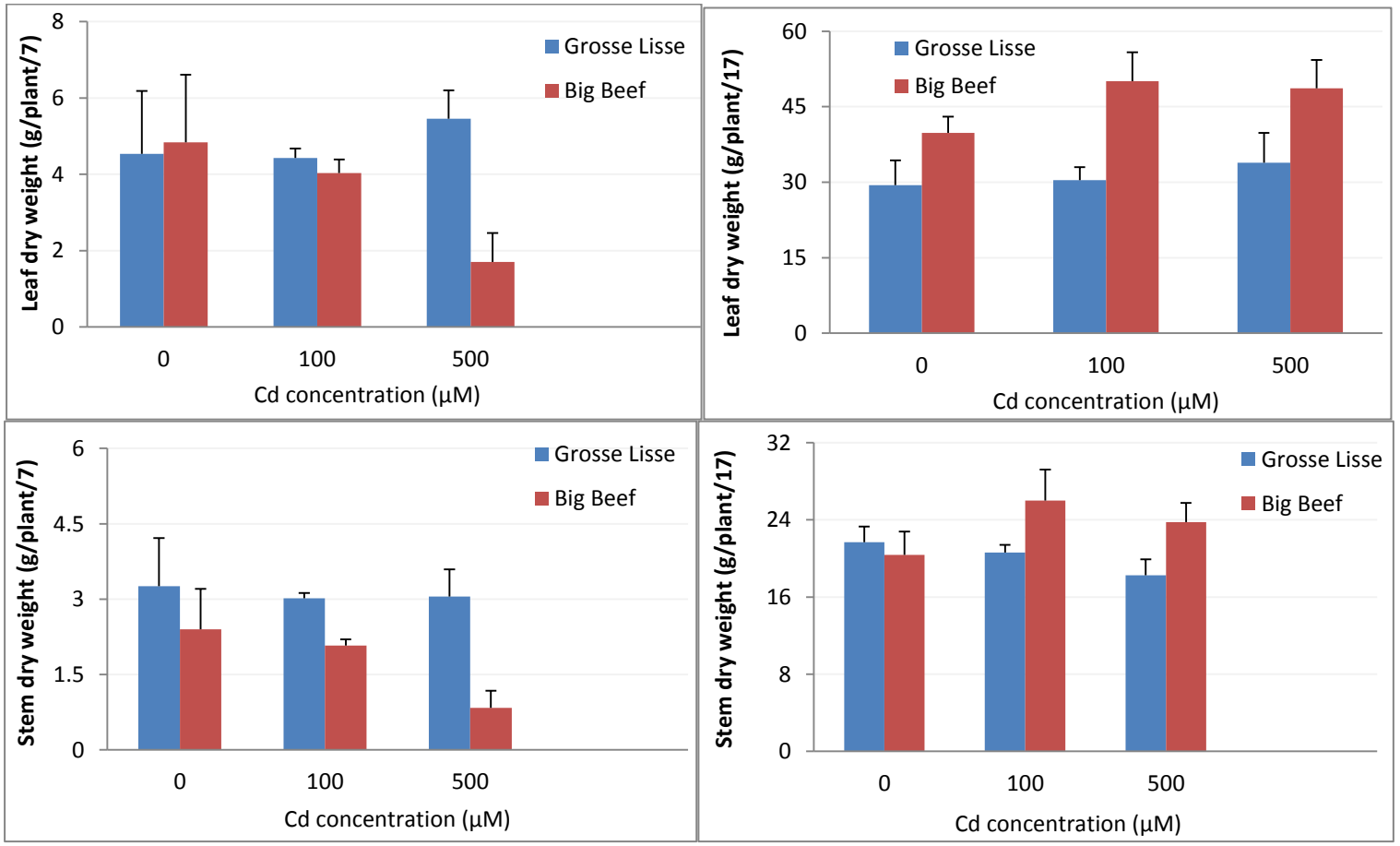


Figure 7.4 Leaf and stem dry weight of 2 genotypes of tomato at 7 weeks (left) and 17 weeks (right) of harvesting. Bars represent SE (n=3).

7.3.5 Effect of cadmium on chlorophyll content, stem girth, number of branches and flower bunches, number of nodes and inter node length

Chlorophyll content was slightly higher in Grosse Lisse than in Big Beef and chlorophyll content was not affected by Cd treatment (Fig. 7.5).

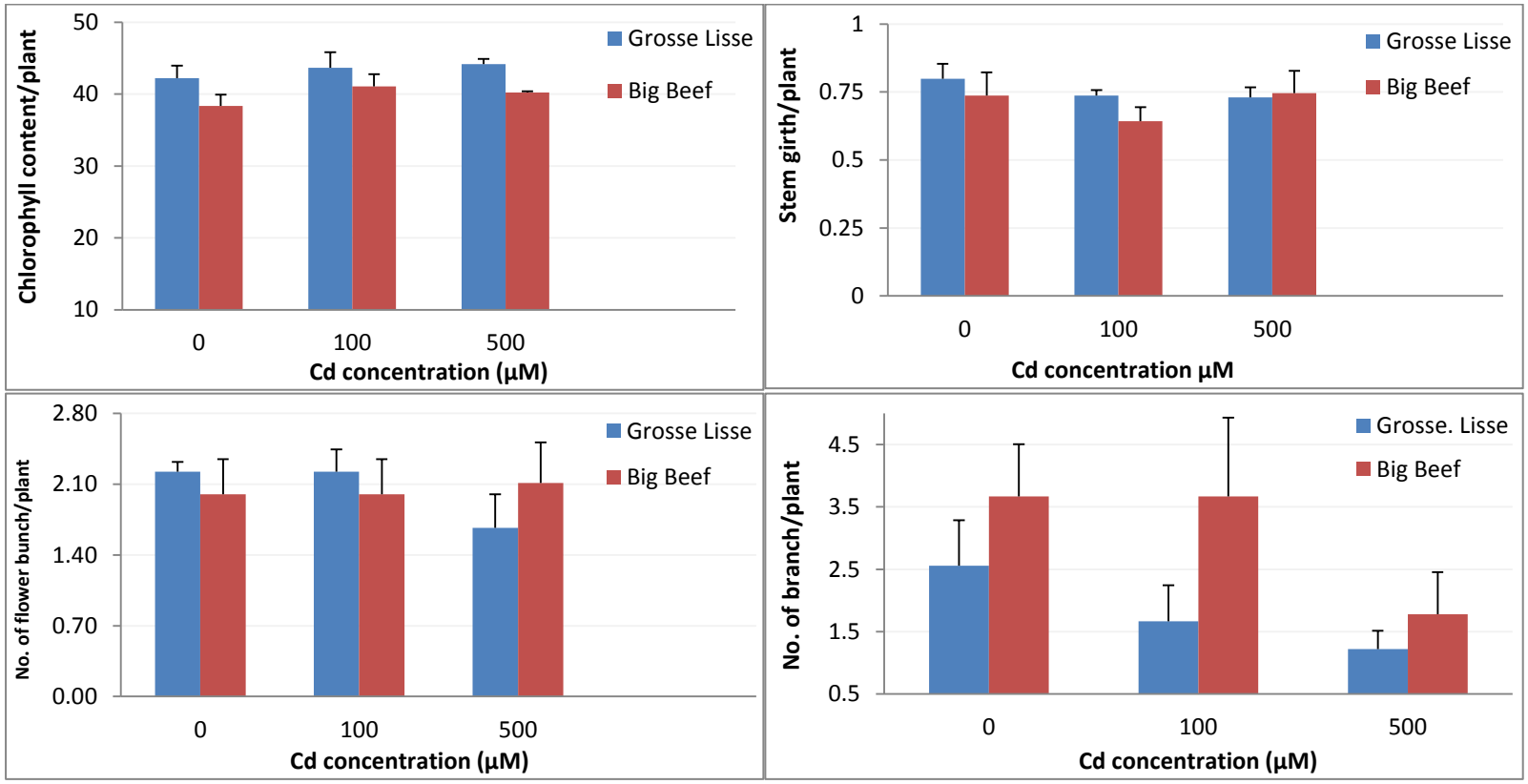
There was no difference between the two genotypes and the Cd treatments in stem girth (Fig. 7.5).

The number of flower bunches decreased in Grosse Lisse at 500 µM Cd as compared to control, but Cd had no effect in Big Beef (Fig. 7.5).

The number of branches decreased in Grosse Lisse and Big Beef at 500 µM Cd as compared to control (Fig. 7.5).

The cadmium did not affect the number of nodes in Big Beef and Grosse Lisse. There was no significant difference between two genotypes and Cd treatment (Fig. 7.5).

The length of internodes was not affected by Cd in both genotypes (Fig. 7.5).


Chlorophyll content/plant
50
40
30
20
10
0
100
500
Cd concentration (µM)
Grosse Lisse
Big Beef
Stem girth/plant
1
0.75
0.5
0.25
0
0
100
500
Cd concentration µM
Grosse Lisse
Big Beef
No. of flower bunch/plant
2.80
2.10
1.40
0.70
0.00
0
100
500
Cd concentration (µM)
Grosse Lisse
Big Beef
No. of branch/plant
4.5
3.5
2.5
1.5
0.5
0
100
500
Cd concentration (µM)
Grosse. Lisse
Big Beef

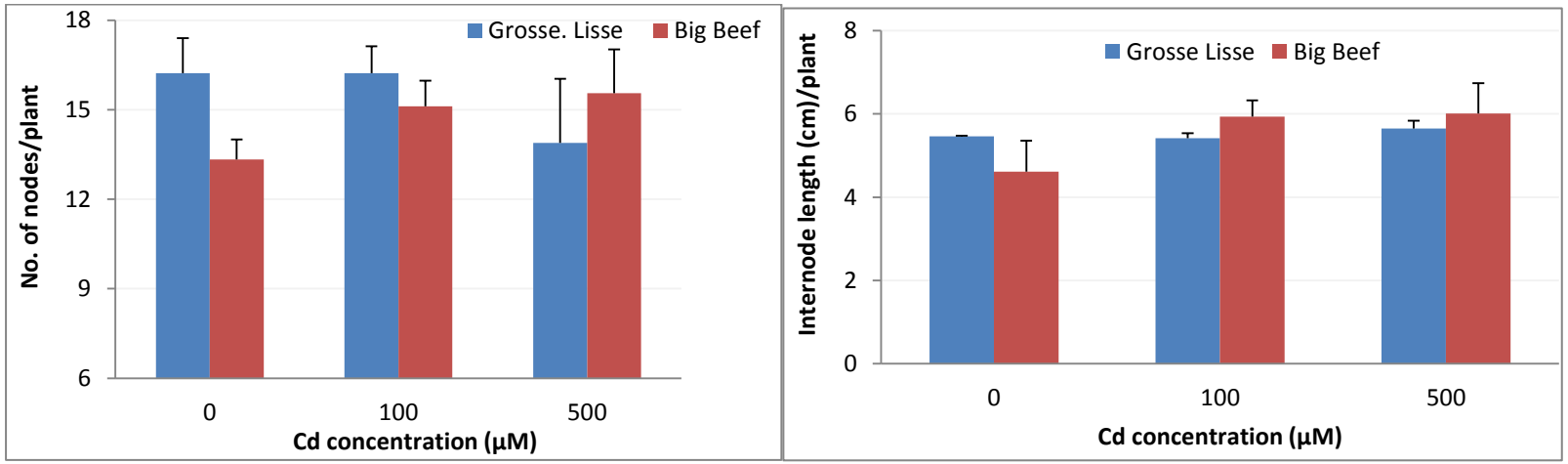


Figure 7.5 Chlorophyll content, stem girth, number of branches and flower bunch, number of nodes and inter node length in 2 genotypes of tomato after 16 weeks of exposure to Cd.

Bars represent SE (n=3).

7.3.6 Toxicity symptoms

No differences in foliar symptoms were observed in the aerial parts of the genotypes Grosse Lisse and Big Beef at 100 and 500 µM Cd. However, the older leaves changed to purple colour in both genotypes at 100 and 500 µM Cd after 17 weeks (Plate 7.2).

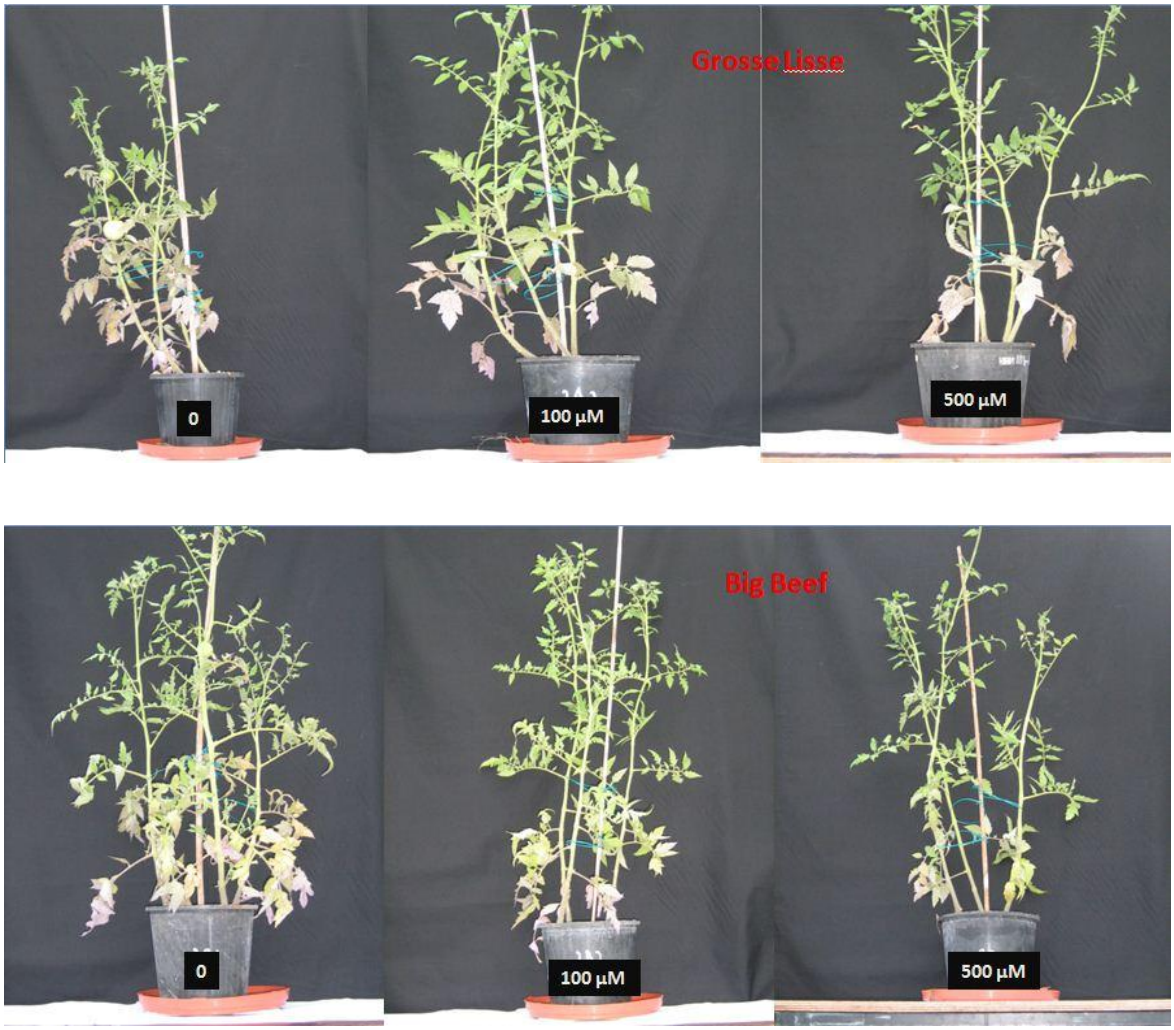


Plate 7.2 Foliar symptoms of genotypes Grosse Lisse (top) and Big Beef (bottom) in control and Cd treated plants.

7.3.7 Leaf cadmium concentration

The leaf Cd concentrations significantly (P<0.001, Table 7.1) increased with an increase in substrate Cd. The leaf Cd concentrations were very high in Big Beef compared to Grosse Lisse at 500 µM Cd. The increment was highest (366 µg/g) in Big Beef and the lowest (223 µg/g) in Grosse Lisse at 500 µM Cd after 7 weeks of exposure (Fig. 7.6).

The leaf Cd concentrations were up to 2 times higher in Grosse Lisse than in Big Beef at 500 µM Cd but there are only small difference between the genotypes at 100 µM. The highest leaf Cd concentration (184µg/g) was found in Grosse Lisse and the lowest (72µg/g) was in Big Beef after 17 weeks of exposure to Cd (Fig. 7.6).

7.3.8 Stem cadmium concentration

The stem Cd concentration was not different between in 2 genotypes at 100 and 500 µM Cd after 7 weeks of exposure to Cd (Fig. 7.6). The stem Cd concentration was similar between the two genotypes at 17 weeks (Fig. 7.6).

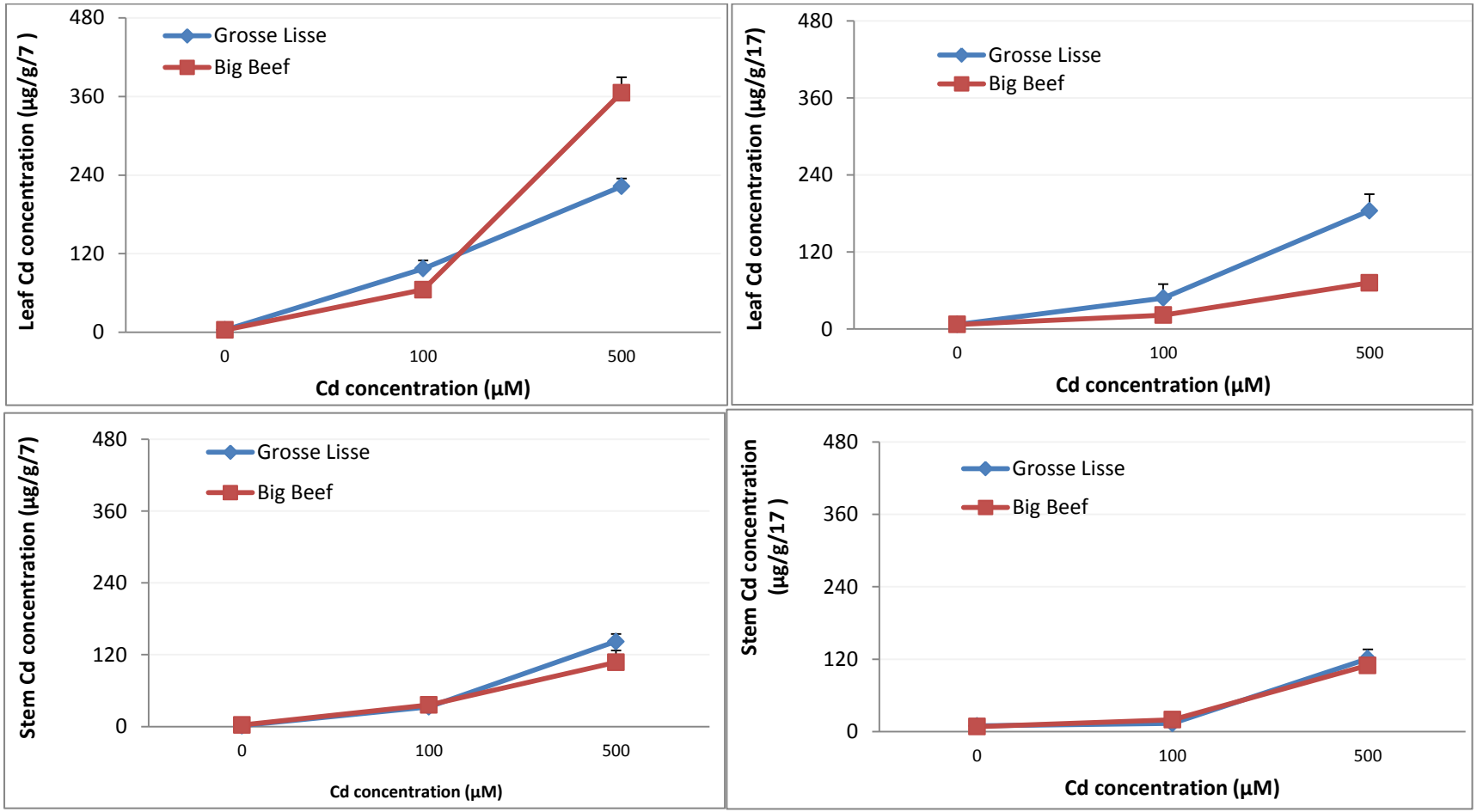


Figure 7.6 Leaf, stem Cd concentration (µg/g) at the three levels of Cd (0, 100, 500 µM) after 7 and 17 weeks of exposure to Cd. Bars represent SE (n=3).

7.3.9 Leaf cadmium uptake

The leaf Cd uptake increased with an increased in the substrate Cd concentration. The leaf Cd uptake was up to 2 times higher in Grosse Lisse than in Big Beef at 500 µM Cd. The highest leaf Cd uptake (1202 µg/plant) was found in Grosse Lisse and the lowest (594 µg/plant) was in Big Beef after 7weeks of exposure to Cd. There was a significant (P<0.05) difference between the genotypes Grosse Lisse and Big Beef and the Cd treatments (Fig. 7.7).

After 17 weeks of Cd exposure, the leaf Cd uptake increased in both genotypes at 500 µM Cd. The highest leaf Cd uptake (5949 µg/plant) was found in Grosse Lisse and the lowest (3513 µg/plant) was observed in Big Beef after 17 weeks of exposure. There was a significant (P<0.05; Table 7.1) difference between the genotypes at 500 µM but not at 100 µM (Fig. 7.7).

7.3.10 Stem cadmium uptake

The stem uptake increased up to 4 times in Grosse Lisse than in Big Beef at 500 µM Cd after 7 weeks of exposure to Cd. However, the uptake did not increase in Big Beef at 100 and 500 µM (Fig. 7.7).

The stems of genotype Big Beef had more Cd uptake (2648 µg/g) than that of Grosse Lisse (2241 µg/g) at 500 µM Cd. The stem Cd uptake was up to 6 times higher in both genotypes at 500 µM Cd than at 100 µM after 17 weeks of exposure to Cd. However, the stem cadmium uptake was not different between the two genotypes at 500 µM and at 100 µM Cd (Fig. 7.7).

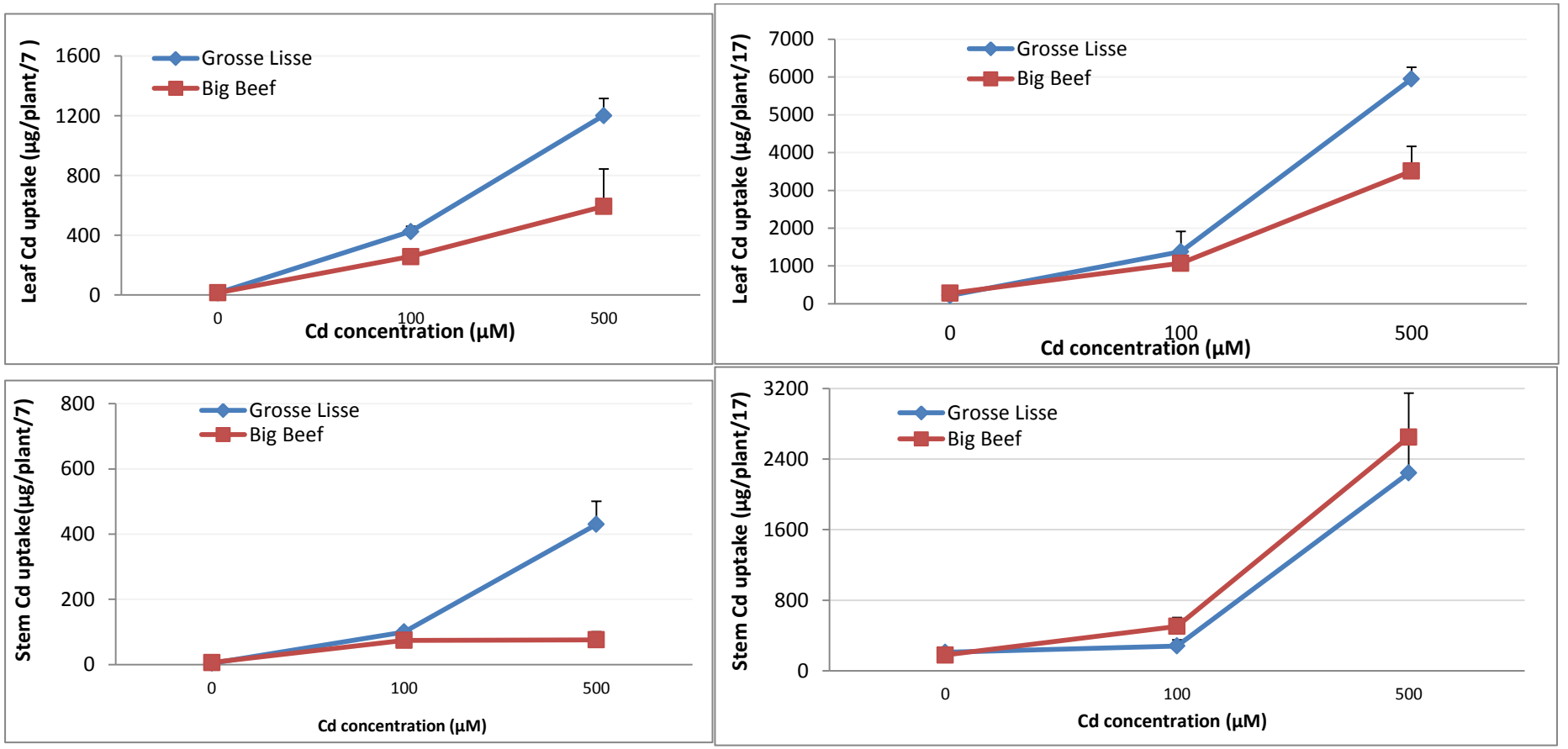


Figure 7.7 Leaf and Stem Cadmium uptake (µg/plant) in 2 genotypes after 7 and 17 weeks.
Bars represent SE (n=3).

7.3.11 Shoot cadmium concentration in two genotypes

There were no significant difference between the two genotypes at 100 µM Cd. Highest shoot Cd concentration (964 µg/g) was found in Big Beef at 500 µM Cd as compared to Grosse Lisse (698 µg/g) (Fig. 7.8).

The shoot Cd concentration increased in both genotypes at 500 µM Cd and there were no significant differences between the two genotypes at 100 µM Cd after 17 weeks (Fig. 7.8).

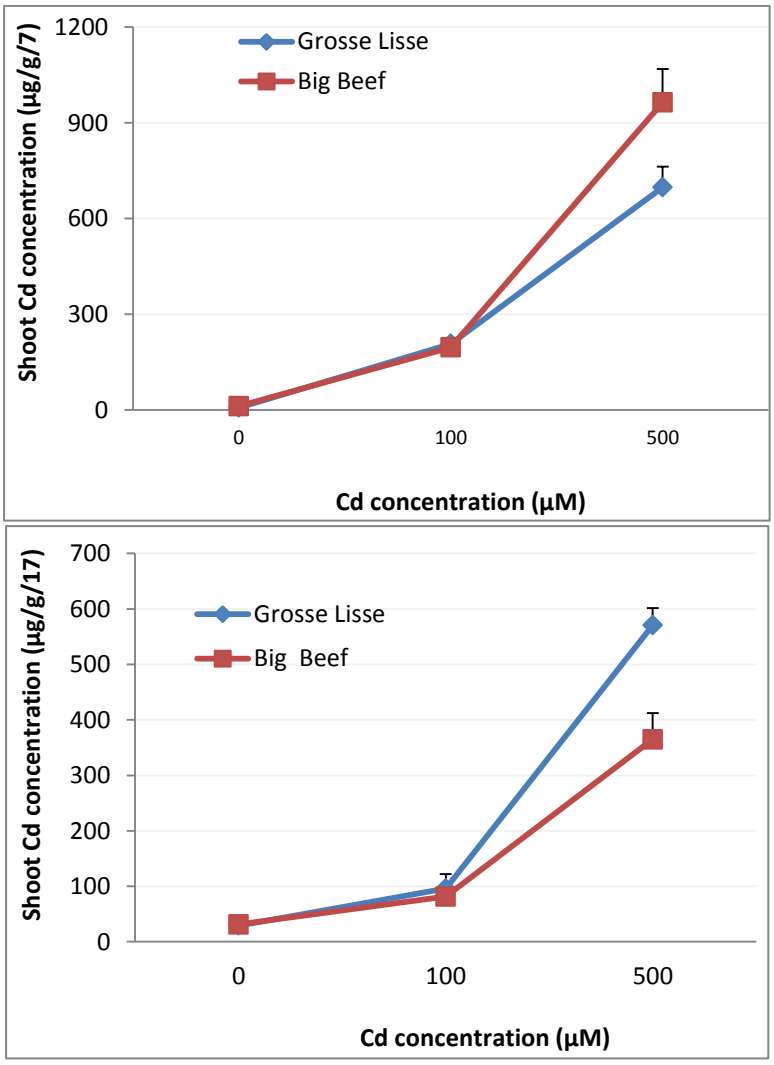


Figure 7.8 Shoot Cd concentration in 2 genotypes at 7 weeks (top) and 17 weeks (bottom)

7.3.12 Shoot cadmium uptake in two genotypes

Shoot Cd uptake was not different amongst the two genotypes after 7 or 17 weeks at 100 µM Cd but it they differed at 500 µM Cd significantly (Fig. 7.9).

The shoot Cd uptake increased up to 4 times in both genotypes at 500 µM Cd as compared to 100 µM. There was no difference between the two genotypes at 100 and 500 µM Cd after 17 weeks of exposure to Cd (Fig. 7.9).

7.3.13 Shoot Biological Absorption Coefficient (BAC)

The shoot BAC value was higher at 500 µM Cd than at 100 µM Cd in Big Beef. The genotypes differed in their BAC except at 500 µM Cd at 7 weeks (Fig. 7.9).

The higher shoot BAC value 170 was found in Grosse Lisse and Big Beef at 500 µM Cd as compared to 100 µM after 17 weeks of exposure to Cd (Fig. 7.9).

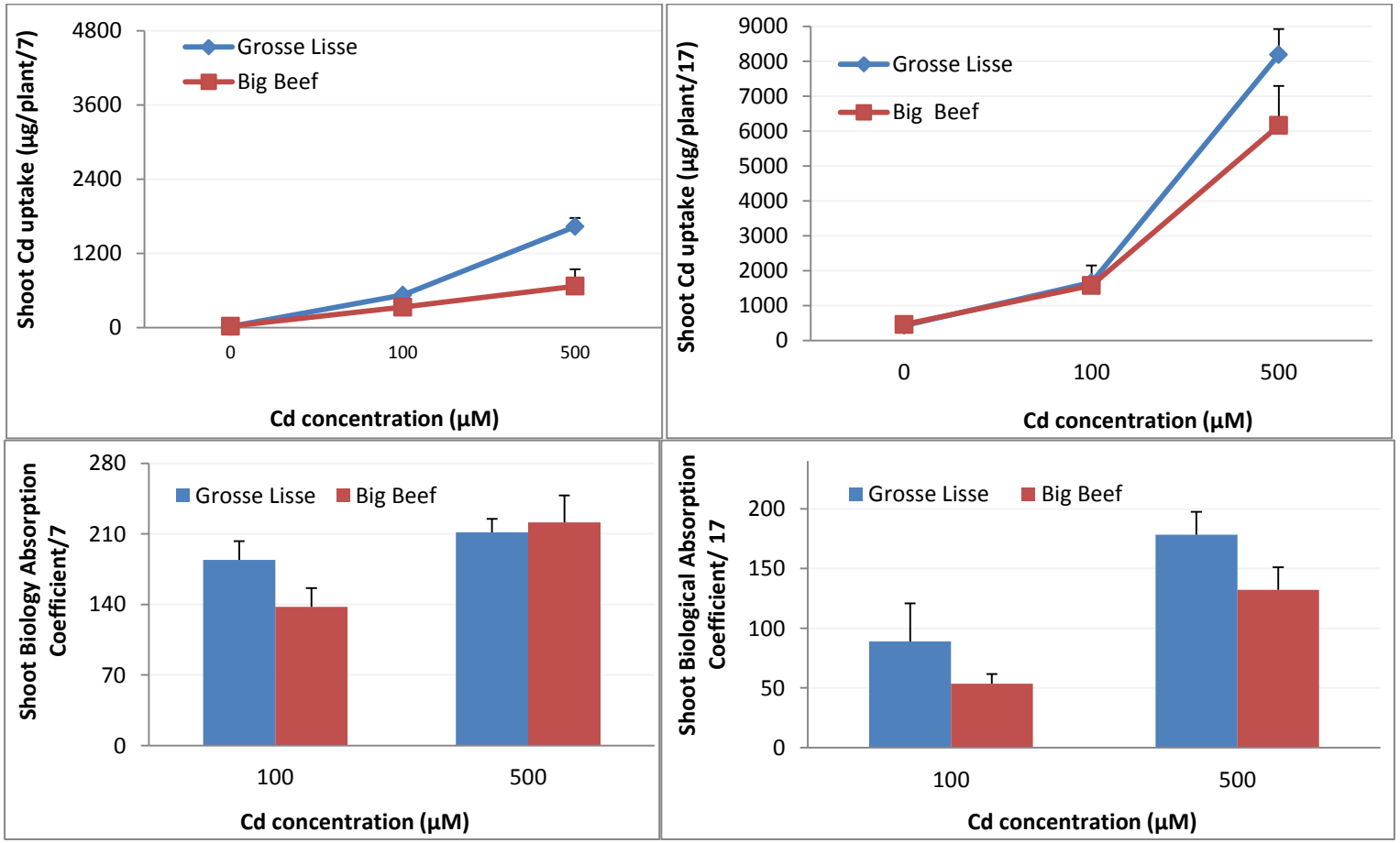


Figure 7.9 Shoot cadmium uptake and Shoot BAC value in 2 genotypes after 7 and 17 weeks of exposure to Cd.

Table 7.1 *P* values from ANOVA for 2 genotypes

	df	Chl con	Fr Dw	Flo bran	Flo bun	Fr FW	G%	17LDW	No of Fr	No of L	Pt ht	17SDW	S girth
Cultivar	1	0.015 *	0.243 NS	0.025 *	0.844 NS	0.210 NS	0.533 NS	0.061 NS	0.241 NS	0.065 NS	0.798 NS	0.054 *	0.298 NS
treatment	2	0.329 NS	0.183 NS	0.046 *	0.701 NS	0.249 NS	0.328 NS	0.667 NS	0.111 NS	0.123 NS	0.045 *	0.374 NS	0.362 NS
Cultivarv*treatment	2	0.874 NS	0.141 NS	0.470 NS	0.541 NS	0.153 NS	0.100 NS	0.134 NS	0.177 NS	0.062 NS	0.253 NS	0.144 NS	0.574 NS

	df	internode Length	No . Node	17L Cd con	17L Cd uptake	17S Cd con	17S Cd uptake	17Shoot uptake	17Shoot con	7LDW	7SDW	7LCdcon	7LCduptake
Cultivar	1	0.981 NS	0.497 NS	0.003 ***	<.005***	0.777NS	0.317 NS	0.052 *	0.002**	0.140 NS	0.012 *	0.008 **	0.025*
treatment	2	0.260 NS	0.742 NS	<.001***	<.001***	<.001***	<.001***	<.001***	<.001***	0.545 NS	0.281 NS	<.001***	<.001***
Cultivarv*treatment	2	0.334 NS	0.281 NS	0.008 **	0.010*	0.611 NS	0.649 NS	0.152 NS	0.024 *	0.136 NS	0.392 NS	<.001***	0.074 NS

	df	7S Cd conc µg/g	7S Cd uptake	7sht uptake (ug/pl)	shoot Conc (ug/g)
Cultivar	1	0.271 NS	<.001***	0.005**	0.089 NS
treatment	2	<.001***	<.001***	<.001	<.001
Cultivarv*treatment	2	0.185 NS	<.001***	0.011*	0.058 NS

F probability; ≥0.05=NS, ≤0.05-0.01=S*, ≤0.01=S**, ≤0.001=S***

S= Significant

NS= Cd did not affect

Df (n-1)=degree of freedom, Chl-con=chlorophyll content,Fr DW=flower dry weight, Flo bran=flower branches, Flo bun=flower bunches, Fr FW=flower fresh weight, G%=germination percentage, LDW=leaf dry weight, No of Fr=number of flower, No of L=number of leaf, Pt ht=plant height, SDW=stem dry weight, S girth=stem girth.

7.4 Discussion

The toxic effects of Cd on plant morphological and physiological processes have been studied using phytoremediation technique in two genoypes of tomato treated with three concentrations of Cd in sand culture.

The results showed that highest seed germination (100%) in Grosse Lisse at 100 and 500 µM Cd and in Big Beef at 100 µM Cd compared to the control. No significant difference was observed between the two genotypes in seed germination. However, some crops are sensitive to high Cd levels. Rumaih *et al*. (2001) reported that the seed germination decreased in cowpea (*Vigna unguiculata*) treated with 218 µM, 436 µM and 873 µM $CdCl_2$ compared to control. Kiran and Sahin, 2006 stated that the seed germination decreased (23%, 37.5% and 68.3%) in lentil (*Lens culinaris*) at 250, 500 and 1000 µM Cd respectively compared to control. Munzuroglu and Zengin (2006) reported that the barley seed germination was inhibited and decreased at 500-10,000 µM Cd when compared to control. According to Farooqi *et al*. (2009), the seed germination of *Albizia lebbeck* decreased at 10, 30, 50, 70 and 90 µM cadmium nitrate as compared to control.

The present study, focussed on the growth of two genotypes capable of tolerating high and low Cd concentrations. The plant height increased in Big Beef at 100 µM and 500 µM Cd compared to control but Cd had no effect in Grosse Lisse. Dong *et al*. (2005) stated that the plant height of tomato cvs Hezuo 903 and Jiangshu 14 was not different at 0.1 µM Cd but decreased at 500 µM Cd after 33 days of exposure to Cd. Cho and Park (1999) found that the plant height increased in tomato (*Lycopersicon esculentum*) treated with 10 µM Cd but decreased at 100 µM Cd. The barley plant height was not inhibited at 1 µM Cd at pH 6.5, but it had slightly

stimulated in plant growth (Guo *et al*. 2004). The plant height decreased in switchgrass at 77 µM $CdSO_4$ (Reed *et al*. 1999). The plant height decreased in *Sedum alfredii* at 200 to 800 µM Cd as compared to control (Yang *et al.* 2004).

In this current experiment, cadmium did not affect the leaf numbers in Grosse Lisse at 500 µM Cd. No significant difference was found between the two genotypes in number of leaves. Dong *et al*. (2005) stated that 32% of the leaf number decreased in tomato cultivar Hezuo 903 and Jiangshu 14 at 10 µM Cd after 33 days of exposure when compared to control. The number of leaves decreased in tomato *Solanum lycopersicum* at 250 µM Cd (Delperee & Lutts 2008).

These results showed tomato fruits to be sensitive to cadmium. No fruits were developed in Big Beef at 100 µM and Grosse Lisse at 500 µM Cd. Very few fruits were observed in Big Beef at 500 µM and Grosse Lisse at 100 µM. The number of fruits showed no significant difference between the two genotypes. There was no significant difference between the two genotypes in dry weight of fruit. Rehman *et al*. (2011) reported that the fruit number of tomato cultivar Navodaya increased when treated with 40 µM Cd when compared to control.In this study, the large stem girth was observed in Big Beef at 500 µM Cd as compared to Grosse Lisse but there was no significant difference between the two genotypes in stem diameter. The cadmium did not affect the length of internodes in both genotypes at 100 and 500 µM Cd compared to control, and no significant difference was observed between the two genotypes with respect to internode length. Djebali *et al*. (2010) stated that stem diameter and length of internode decreased in *Solanum lycopersicon* at 100 µM Cd compared to control. The stem diameter decreased in *Zygophyllum fabago* at 10 µM Cd compared to control (Lefevre *et al.* 2005).

In this experiment, there was no significant difference between the two genotypes in number of nodes produced. Lefevre *et al.* (2005) reported that the number of nodes was not reduced but the development of axillary buds was inhibited at 10 µM Cd in *Zygophyllum fabago* compared to control after four weeks of exposure to Cd.

In the present work, the leaf and stem dry weight was not significantly different in Grosse Lisse but decreased in Big Beef at 500 µM Cd as compared to the control after 7 weeks. The leaf dry weight of tomato was higher than stem dry weight after 7 and 17 weeks. There was no significant difference between the two genotypes for leaf dry weight after 17 weeks. Lopez-Millan *et al*. (2009) stated that the leaf and stem dry weight decreased in tomato (*Lycopersicon esculentum*) treated with 100 µM Cd compared to control. The shoot dry weight decreased in *Solanum nigrum* at 100 µg/g Cd compared to control (Sun *et al*. 2006). The leaf and stem dry weight reduced at 7120 µM Cd in tomato compared to control (Cherian *et al*. 2007). The dry weight of shoots increased at 25 µM Cd in *Sedum alfredii* but decreased at 800 µM Cd as compared to control (Yang *et al*. 2004). The shoot weight of *Thlaspi caerulescens* increased at 10 µM Cd but decreased at 20-50 µM Cd compared to control (Keller *et al.* 2006).

In the present study, high chlorophyll content was recorded in Grosse Lisse at 100 µM and 500 µM Cd as compared to Big Beef. Chlorophyll content was significantly different between the two genotypes. Cherian *et al*. (2007) showed that the chlorophyll content decreased in tomato (*Lycopersicon esculentum*) treated with 7120 µM Cd. The chlorophyll content decreased at 1779 µM Cd in corn and sunflower plants compared to control (Pritsa *et al*. 2008). The chlorophyll content reduced at 500 µM Cd in tomato compared to control (Cho and Park 1999).

In the present work, no foliar symptom was observed in aerial parts of either tomato genotype, but the old leaves changed to a purple colour in both genotypes at 100 and 500 µM Cd after 7 weeks of exposure to Cd. Roosens *et al.* (2003) stated that necrosis symptoms were detected at 30 µM Cd in *Thlaspi caerulescens*. Chlorophyll depletion and wilting symptoms were found in tomato plants at 7120 µM Cd (Cherian *et al.* 2007). Symptoms of Cd toxicity were observed in spinach such as necrosis on the tips of cotyledonary leaves and chlorosis and necrosis on primary leaves treated with 0.316 µM of Cd after 10 days of exposure (McKenna *et al.* 1993). Chlorosis symptoms were found in lettuce at 35 µM and 50 µM $CdCl_2$ (Zorrig *et al*. 2010). The leaves of *Sedum alfredii* wilted after four days when the plants were treated with 800 µM Cd. Older leaves began to fall off after 16 days of treatment (Yang *et al*. 2004).

The leaf and stem Biological Absorption Coefficient was higher in Grosse Lisse and Big Beef at 500 µM Cd compared to 100 µM after 17 weeks of exposure to Cd. These two genotypes are suitable for phytoextraction. Tukura *et al.* (2012) reported that the BAC value for cadmium in okra (1.68) was higher than in pepper (1.24) at farm B, Mada River, Nigeria.

In the present experiment, leaf Cd concentration was higher in Big Beef than in Grosse Lisse but leaf Cd uptake was higher in Grosse Lisse than in Big Beef after 7 weeks. Higher leaf Cd uptake was found in Grosse Lisse than in Big Beef after 7 and 17 weeks. The stem Cd concentration was not different in both genotypes after 7 and 17 weeks. However, stem Cd uptake was higher in Grosse Lisse than in Big Beef after 7 weeks. The stem Cd concentration and uptake were not different in both genotypes after 17 weeks. Higher shoot Cd concentration was observed in Big Beef

than in Grosse Lisse after 7 weeks but the shoot Cd uptake was higher in Grosse Lisse than in Big Beef after 7 and 17 weeks. Kashem *et al*. (2008) reported that the highest Cd accumulation was found in arum at 50 µM Cd and in radish and water spinach at 10 µM Cd compared to control. The shoot Cd contents increased sharply at 50 µM $CdCl_2$ in the three cultivars of lettuce compared to control (Zorrig *et al*. 2010). The Cd uptake increased at 214 µM Cd in root, shoot and grain of pea when compared to control (Wani *et al.* 2008).The Cd content and Cd accumulation decreased at 1 µM and 5 µM Cd in the shoots of two rice cultivars compared to Cd content and accumulation of root (Zhang *et al*. 2009). Sunflower plants had higher Cd concentrations in roots and lower concentrations in shoots treated with 1779 µM Cd compared to corn (Prista *et al*. 2008). The higher Cd accumulation was observed in tomato (*lycopersicon esculentum*) at 7120 µM Cd compared to control after 14 days of exposure to Cd (Cherian *et al*. 2007). The high Cd accumulation was observed in the shoot and root of *Solauum nigrum* at 0.89 µM Cd compared to control (Sun *et al.* 2006).

In conclusion, the responses of the two genotypes to cadmium varied with the Cd levels. Above 93% seed germination was observed in the two tomato genotypes Big Beef and Grosse Lisse with high Cd levels. High Cd concentrations did not affect plant height of the two tomato genotypes. The number of fruits and fresh and dry weight of fruits were sensitive to high Cd concentrations. It was observed that the stems had a lower Cd uptake than their leaves. The leaf Cd concentration was higher after 7 weeks than at 17 weeks but the leaf and stem Cd uptake was higher after 17 weeks than at 7 weeks of exposure to Cd. The leaves of Grosse Lisse had 2 times higher Cd uptake than Big Beef at 500 µM Cd at 7 weeks and 17 weeks. The stem Cd uptake was 6 times higher in both genotypes after 17 weeks than at 7 weeks in

500 μM Cd. This suggests that the stems may continue to accumulate Cd over time, and longer exposure of plants may lead to greater removal of Cd from the soil.

Chapter 8

Testing the use of Near-Infrared Reflectance (NIR) and X-Ray Fluorescence (XRF) spectroscopy to detect cadmium in tomato tissues

8.1 Background

Metal contamination in soil is one of the major environmental problems. The metals can be translocated from plant roots to shoots and this can affect human health. Thus it is necessary to analyse the tissues for heavy metal concentrations.

Tissue Cd concentration can be determined by various methods such as atomic absorption spectrometry (AAS), inductively coupled plasma (ICP) mass spectrometry, X-ray fluorescence spectrometry and near-infrared reflectance spectroscopy (NIR) (Zhang *et al*. 1998; Anjos *et al*. 2002; Xu *et al*. 2007). Hokura *et al.* (2004) monitored distribution of Cd in *Nicotiana tabacum,* a hyperaccumulator plant, using synchrotron X-ray fluorescence microprobe. The results showed that Cd was found in the protoplast. The high energy micro-XRF imaging has also helped determine the distribution of Cd at sub-cellular levels. The Kα peaks of K, Ca, Mn, Fe, Zn, Rb, Sr, Mo and Cd could be clearly observed in the leaves of tobacco.

Siebielec *et al*. (2004) employed near-infrared (NIR System 6500) and mid-infrared (DigiLab FTS-60 Fourier Transform Infrared Spectrometer) diffuse reflectance spectra to estimate total Fe, Cd, Cu, Ni and Zn content of soil in the mining affected grazing land of the Tarnowskie Gory region in Poland. Calibration model performance of the NIR system 6500 spectra (wavelength range 400 to 2500 nm) was less accurate (i.e. R^2<0.9) compared to FTIR spectrometer for all trace heavy elements included in the study. Models were developed using the one-out validation

procedure Partial least squares regression wherein iron, Cd, Cu, Ni and Zn were successfully predicted.

In this study two techniques were used, viz., NIR spectroscopy and XRF spectroscopy to detect Cd concentrations in the tomato stem tissues.

8.2 Near Infrared Reflectance Spectroscopy

Conventional methods of determining metals in plant tissues rely upon wet digestion of dried samples using an acid and analysing the metal concentration of the digested material using atomic absorption spectrometer (AAS). This method is time-consuming, requires chemical reagents, skilled labour and costly analytical equipment (Batten 1998). A rapid, simple, non destructive and cost-effective method for determining heavy metal content of plant tissue would be very useful in environmental studies.

X-ray spectroscopy is one such technology, suitable for assessment of elements above an atomic number of 20 and at concentrations above 0.5 mg/kg. Indeed, instrumentation is available in a hand held form, allowing for field use SIRA manufacturer (Technologies at SIRA are modern and from credible global manufacturers). However, the detection limit of this method is such that it is of use only in severely contaminated plants, presented as dried ground material. For example, the detection limit for Cd is approximately 0.5 mg/kg, while upper limit for Cd in plant tissue is typically 0.1 mg/kg of fresh weight (Senn and Milham, 2007).

The technique of NIR spectroscopy (NIRS) also possesses advantages of minimal sample preparation; with dried ground plant material routinely assessed for protein and sugar content (Batten 1998). The technique relies upon the absorption of

radiation associated with second and third overtones and combinations of stretching of dipolar bonds such as C-H, N-H, O-H and S-H (Golic *et al*. 2005). However, constituents not containing these bonds can be assessed if they consistently impact on the absorption features of other components of the matrix. For example, NaCl content can be assessed using NIRS due to the influence of NaCl on the H bonding of water. Indeed Batten (1998) assessed K content of dried rice tissue. As such, the technique holds potential for the assessment of heavy metals in plant tissues, either by accumulation of an organic compound in proportion to heavy metal content, or by some change in cell size and structure that affects light scattering.

The major considerations for use of NIRS in a particular application are (a) what absorption or scattering feature of the sample is related to the constituent of interest; (b) what depth of penetration is required (greater depth requires use of shorter wavelengths); (c) what sample to noise detector system is required (related to the concentration of the constituent); (d) what sample optics are appropriate (Herold *et al*. 2009). For the assessment of metals at relatively low concentration (i.e. sub percent levels) in plant tissues the use of reflectance spectroscopy with dried, uniformly ground material is logical, as practiced, for example, by Batten (1998).

Near infrared spectroscopy is a non-destructive, rapid and low cost technique (Huang *et al*.2008). This technique works on the principle of either absorb organic molecules by NIR radiation at specific regions or wavelengths (particularly, N-H, O-H and C-H bond) or scattered within the tissue.Several authors have reported the use of NIRS to assess heavy metals associated with sediments, soils or plant material. For example, Malley *et al*. (1996) applied near infrared reflectance spectroscopy (Foss-NIRSystem 6500) to predict organic-bound Cd in lake picoplankton. In this

experiment, 1.5 ml of lake water was filtered through a Whatman GF/C glass fibre filter paper, and dry filter papers were scanned using a NIR Systems 6500 (Foss NIR Systems, Silver Spring, MD) in a reflectance mode over the range of 400-2500 nm. Following spectral acquisition, the Cd present on the filter paper was determined following acid digestion by atomic absorption spectrophotometer. Samples were then sorted by Cd content (based on reference method) and divided in to two groups, separating every two samples from three for use in calibration, and every third sample for validation of the regression model. Validation statistics of n=13, mean=1.14, SD=0.39 and R^2 of 0.75 and SEP of 0.191 were reported. The authors concluded that this technology has good potential to discriminate the nature of the organic matter with which heavy metals (i.e. Cd) are associated. However, their conclusions are likely to be optimistic, given that the prediction set was a sub-set of the calibration set, and the small number of samples used.

Kumagai *et al*. (2003) reported on the assessment of cadmium content, in unpolished rice using a portable NIR spectrometer, PlaScan SH (OPT Research, Inc., Japan) with wavelength ranging 1200-2400 nm and canonical discriminant analysis (CDA). All samples included 106 unpolished rice and were divided into three groups such as low Cd polluted rice (<0.4 ppm Cd), medium Cd polluted rice (0.4-1.0 ppm Cd) and high Cd polluted rice (>1.0 ppm Cd). The NIR spectra predicted low Cd polluted rice was classified 86.7%, medium Cd polluted rice was 83.9% and high Cd polluted rice was 87.5% for 318 NIR spectra, repeated three times for 106 samples. The authors suggested that such results are sufficient to recommend the technique to detect Cd concentration in cadmium polluted rice.Chodak *et al*. (2007) reported the use of a NIR system 6500 in reflectance mode to predict total and exchangeable concentrations of Zn and Pb from the ‘O’ horizon of forest soil in a heavy metal

polluted area. The spectra of soil samples with heavy metal pollution were reported to be different to that of uncontaminated soils using near-infrared spectroscopy. The NIRS technology was reported to quantify total and exchangeable concentrations of Zn and Pb along with other chemicals and microbes from the 'O' horizon of forest soil. The best calibration model was reported for Carbon to Nitrogen bond $R^2 = 0.96$, RMSECV =1.5 % (with a population of mean ± SD = 26.0 ± 4.4 %). However, no attempt was made to validate the model using a set of samples not represented in the calibration process.

Thus prior work on the use of NIRS to assess heavy metal content of plant tissue is not conclusive. The aim of current study was to extend these previous studies, to determine whether it is appropriate to use VIS/NIR spectroscopy, for the assessment Cd content of dried and ground plant tissue of tomato stems.

8.2.1 Materials and Methods

8.2.1.1 Plant culture

A stock solution containing 100 mM Cd was prepared by dissolving 25.6 g of $3(CdSO_4)\ 8H_2O$ (molecular weight 769.52 g) in 1000 ml of water.

Manutec hydroponics nutrient salt purchased from Bunning's Warehouse was prepared according to manufacturer's instructions (72 g of Part 1 fertilizer [N.P.K 7.6/3.1/18.2 + Trace Elements] was mixed in 60 L of Reverse Osmosis (RO) water and then 48 g of Part 2 [calcium nitrate] was added. No cadmium stock solution was added for the control treatment, while 0.3 ml stock solution was added to 60 L of stock to achieve a final concentration of 30 µM Cd, 2 ml for 200 µM, 5 ml for 500 µM and 10 ml for 1000 µM treatments. The pH of the hydroponics solution was

initially adjusted to 6.3 and after addition of $CdSO_4$ solution, the pH was adjusted to 5.3.

Dried sand was weighed (1.5 kg) into each of 60 polyethylene pots (12 x 9 x 12.5 cm) and the pots were placed in a tray (Plate 6.1). The pots and the saucers were placed in non-draining tubes. Ten seeds of a single variety of tomato (*Lycopersicon esculentum* L.) were sown to each pot. Sixty pots were sown for each of four varieties. Each pot was irrigated with 100 ml of RO water. After seedling emergence, five treatments were applied (irrigation with 0, 30, 200, 500 and 1000 µM Cd) to each of three replicate pots per genotype. Hydroponics nutrient solution containing with and without Cd was added to respective treatment pots at 200 ml/pot during the first week and 120 ml/pot during the second week. The experiment was run in a glasshouse which was maintained at a temperature of 25-32°C using an evaporative cooler.

8.2.1.2 Sample preparation for reference analysis

Three weeks after imposition of the Cd treatments, three seedlings from each pot were harvested and washed with demineralised water. The shoots were separated into leaves and stems, and stem tissue dried in a fan forced oven at 72°C over 3 days. Plant samples were ground using a Culatti hammer mill (MFC Mikro-Feinmuhle-Culatti) equipped with a 1.5 mm sieve. A sample of the resulting powder (0.4 g) was placed into 10 ml PPTR plastic tube and 2 ml of HNO_3 and one drop of H_2O_2 was added. The tubes were held overnight in a fume hood. On the following day, 3 ml of Milli-Q water was added to each tube, and the tube held in the water bath (Grant Instruments, Cambridge Ltd) at 70°C for 4 hours. After digestion, a further 5 ml of Milli-Q water was added, and the tubes were then centrifuged at 15,000 gfor 15

minutes. The Cd concentrations of the supernatant was analysed by using Atomic Absorption Spectrophotometer (Varian Australia Pty. Ltd). The Cd concentration of four genotypes of tomato is presented in Table 8.1.

Table 8.1 Cadmium concentration in the stem samples of four genotypes of tomato.

Cultivar	SDW(g)	µg/g
Big Beef	5.279	7.50
	5.465	15.40
	5.111	49.60
	5.166	86.70
	3.991	129.00
Mean	5.00	57.64
SD	0.58	50.74
Tiny Tom	3.385	6.80
	3.793	12.20
	3.319	54.80
	3.986	73.10
	1.158	143.20
Mean	3.13	58.02
SD	1.14	55.26
Burke's Backyard	4.222	4.40
	3.886	7.90
	4.608	28.80
	3.136	64.80
	3.643	117.70
Mean	3.90	44.72
SD	0.56	47.34
Grosse Lisse	3.077	4.80
	3.967	14.00
	4.435	77.20
	5.064	96.00
	5.95	88.30
Mean	4.50	56.06
SD	1.09	43.24

8.2.1.3 Acquisition of NIRS spectra

Ground oven dried material which was used in acid digestion procedure was stored in airtight containers. These samples were re-dried at 65°C for 72 hours, prior to transferring to NIRSystem spinning cups. Three backing plates and compression springs were used with each spinning cup to obtain a similar packing density. The time between transfer of sample from a container to a spinning cup and subsequent spectra acquisition was kept to a minimum time interval to avoid moisture absorption by the sample.

Reflectance spectra were obtained of the ground stem samples using a NIR Systems Model 6500 (Foss NIR Systems, Silver Spring, MD). The spectra were collected every 2 nm at a resolution of 10 nm over the wavelength range 400 and 2500 nm. Duplicate spectra of samples were acquired.

8.2.1.4 Chemometrics and statistics

The chemometric software package, The Unscrambler V. 9.1 (Camo), was used for partial least squares regression (PLSR) calibration model development (prediction of a given attribute from spectral data). Calibration model performance was assessed in terms of correlation coefficient of determination of calibration sets (R_c^2), root mean squares error of calibration (RMSEC), root mean squares error of cross-validation (RMSECV) and standard deviation ratio (SDR) = (SD/RMSECV), while performance in validation was considered in terms of R_p^2, root mean squares standard error of prediction (RMSEP), SDR, slope and bias of validation sets. Cross-validation was performed using full-cross validation of samples. An in house package developed in Mat lab (Guthrine *et al*. 2005) was used to compose PLS models developed using different wavelength regions.

8.2.2 Results and Discussion

8.2.2.1 Optimisation of spectral window for calibration model development

In this study, near infrared analysis was applied to determine the cadmium concentrations of dried stem tissues of tomato that were exposed to cadmium for up to 7 weeks in sand culture (see Chapter 6). The tissue concentrations were calculated from NIR spectra using Unscrambler chemo metrics statistical software. Figure 8.1 demonstrates reflectance spectra (log 1/R) and second derivatives spectra (d^2 log 1/R) of dried ground samples of tomato stems. There was no obvious relationship between log 1/R of d^2 log 1/R spectra of Cd level in the sample (from visual inspection of Fig. 8.1). The log 1/R plot demonstrated a baseline increase at higher wavelengths, expected for reflectance spectra of a ground sample (scattering phenomenon). This texture was effectively removed by the second derivative pretreatment. Peaks at 1400- 2200 nm are essential to residual matter in the sample.

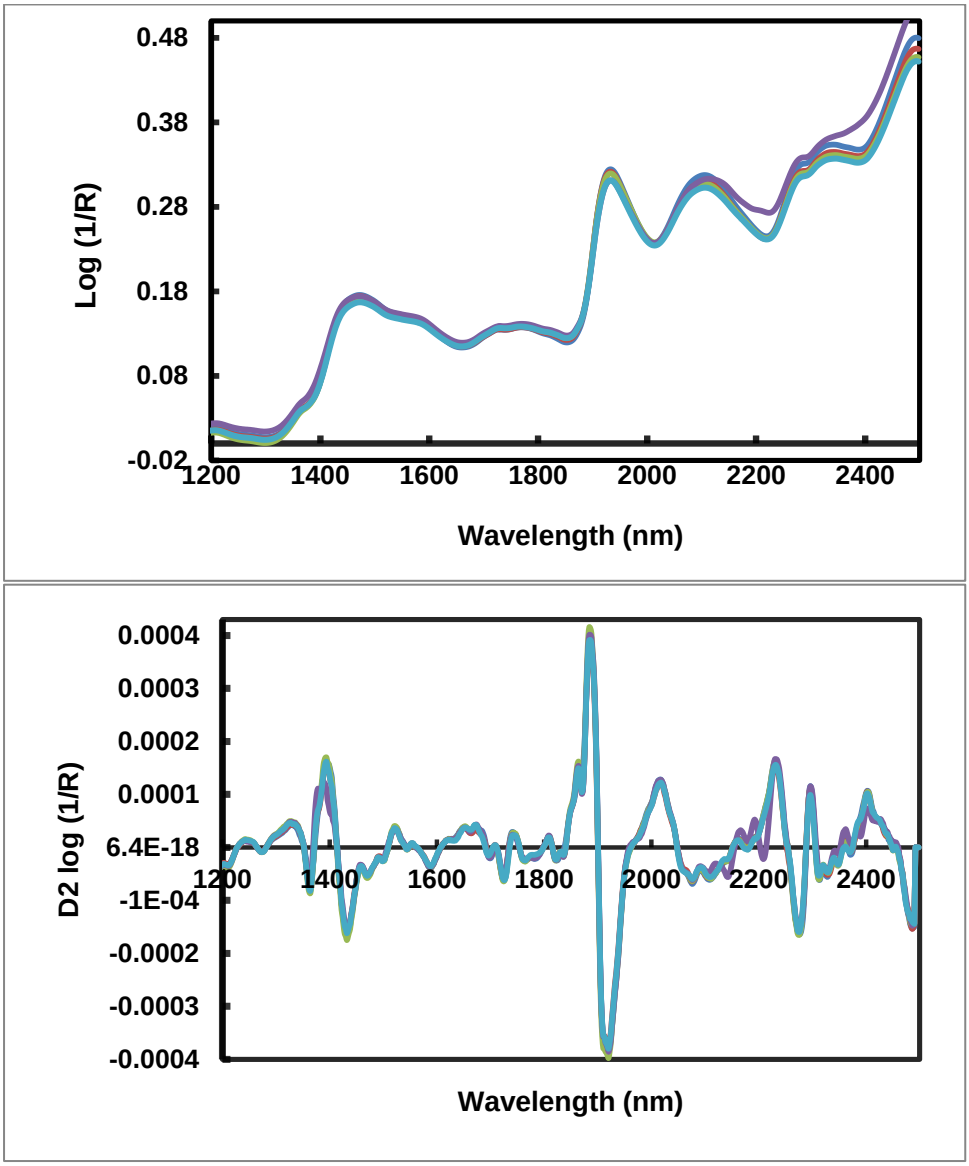


Figure 8.1 Top panel reflectance (log 1/R) spectra and bottom panel second derivatives spectra (d^2log 1/R)

Given the effectiveness the second derivative d^2 log (1/R) spectra for the calibration. In the second derivative spectra, the base line shift of the spectra seemed to be corrected approximately. The differences in peak intensity of Cd concentration at 1850 nm and 1950 nm could be observed. The best calibration models was made by comparing statistical parameters that were calculated by the software. The NIR method cannot be more sensitive than its primary analytical method, it was important that the SE of the NIR method be at least as good as that of the reference method.

Khuriyati *et al*. (2004) stated that NIR spectra of tomato fruits were detected with a commercially available spectrometer, model MMS1 in the spectral window of 305-1100 nm. The results showed second derivative spectra of tomato was different in peak intensity of the water band at 974 nm could be seen.

8.2.2.2 Calibration and validation window

In house built software was used to identify appropriate spectral window to develop PLSR models for all possible ranges within a set wavelength region. Model number of factors, RMSEC and RMSEV were used to select the best wavelength region. As shown in Fig. 8.2 low RMSECV was achieved with a start around 500 nm and a finish up to 1350, or 1850 or 2300, or a start at around 950 and a finish around 1300, or a start around 2100 and a finish around 2300 nm. There was a similar number of factors in each of these cases (around twelve), except for the 500-1000 nm window, which used fewer factors (around 10). The use of vis-short wave window of 500-1000 nm is likely to involve weighting of the chlorophyll peak, with Cd treated stems having lower chlorophyll. The window 500-2500 nm was used for further prediction.

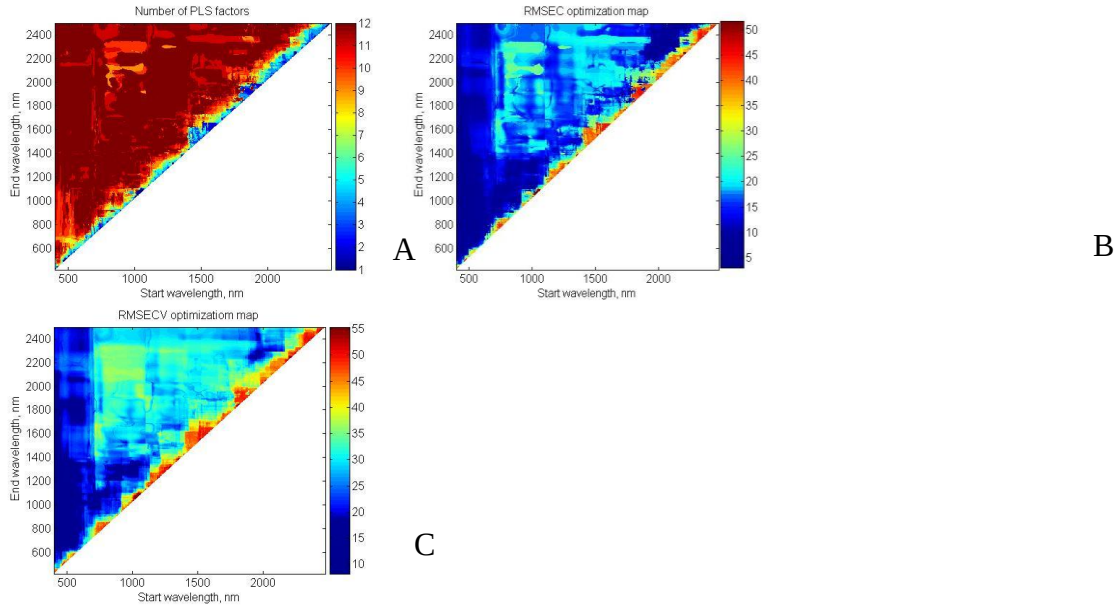


Figure 8.2 Optimisation of calibration and validation window: optimum number of PLS factors (A), RMSEC and RMSECV for all combinations of starts and stop wavelengths between 500 and 2500 nm respectively (B) and (C).

8.2.2.3 Analysis of partial least square loading

The PLS loading spectra for factors 1 and 2 (Fig. 8.3) revealed the presence of several groups based on Cd concentrations. Two dimensional (2D) PLS loadings component score plot using the first two score vectors, PC1 and PC2, derived from log 1/R or d^2 log 1/R of the samples. Two separated cluster PC1 and PC2 are observed. These two principle components were explained 94% of spectral variance in X axis (Fig. 8.3). The PLS loading gives important information about the basic data structure, regarding a potential capability of separation of objects. Loading indicates the visualising dimension spaces, different classification methods were utilised for an improved separation. However, first two score vectors indicated that there was no clear classification based on Cd concentration revealed that given the number of samples and latent variables used for calibration model development might have effect of model over fitting. However, Xie and Ying (2009) stated that principal component analysis was used to detect a potential capability of separating

in tomato juice using NIR spectra OMNIC software at a range from 800-2400 nm. Negative scores were found in dataset (A) of the first component but positive scores were found in dataset (B) of the first component.

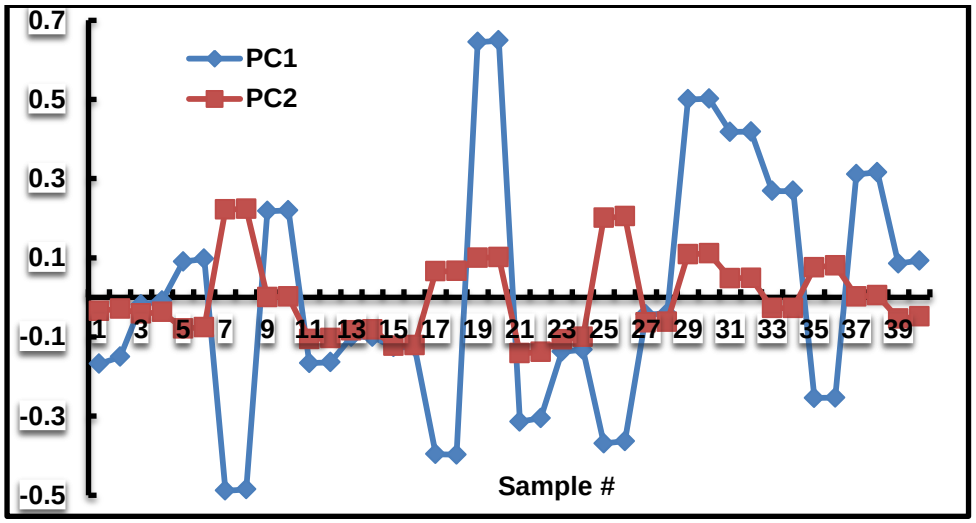


Figure 8.3 Partial least square regression two dimensional loading principle component one based on Cd concentration

8.2.2.4 Partial least square (PLS) model

PLS model development was undertaken using The Unscrambler chemomatric software using the window 500-2300 nm, RMSEC and RMSECV were minimised with use of 12 or more Latent Variables (Fig. 8.4).

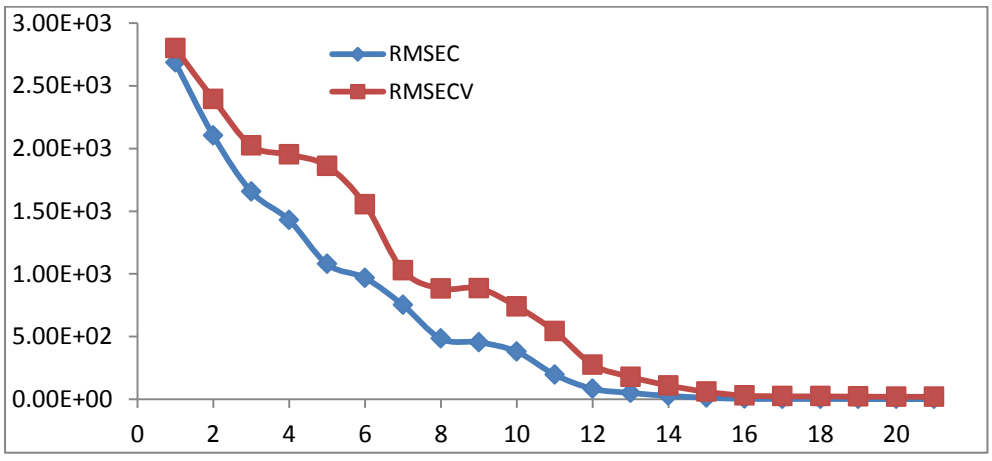


Figure 8.4 Optimisation of Latent Variables for calibration model development within the spectral range 500 nm- 2300 nm.

8.2.2.5 PLS model performance

Twenty dried ground samples of tomato stems that were exposed to cadmium in sand culture (4 genotypes (2 g DW) x 5 Cd concentrations) were scanned in the NIR System 6500 visible-NIRS in the reflectance mode over a wavelength range from 400-2500 nm. Fig. 8.5 and 8.6 showed partial least squared (PLS) regression calibration model of cadmium concentration of dried ground stems of tomato plant and PLSR cross validation statistics respectively, for 20 tomatoes stem samples containing Cd. In both figures, predicted cadmium concentrations and measured cadmium concentrations are highly correlated. The best correlation coefficient of determination R_c^2=0.9989 and R_v^2=0.989 were observed. These results indicated that NIR technology can be used to predict cadmium concentrations of tomato stem tissues.

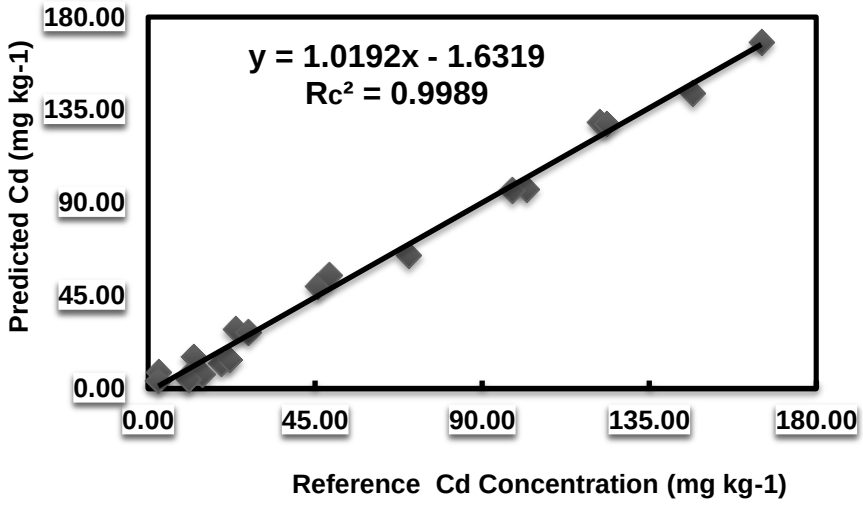


Figure 8.5 Calibration Partial Least Square Regression model performance of variance concentrations on 4 genotypes of tomato.

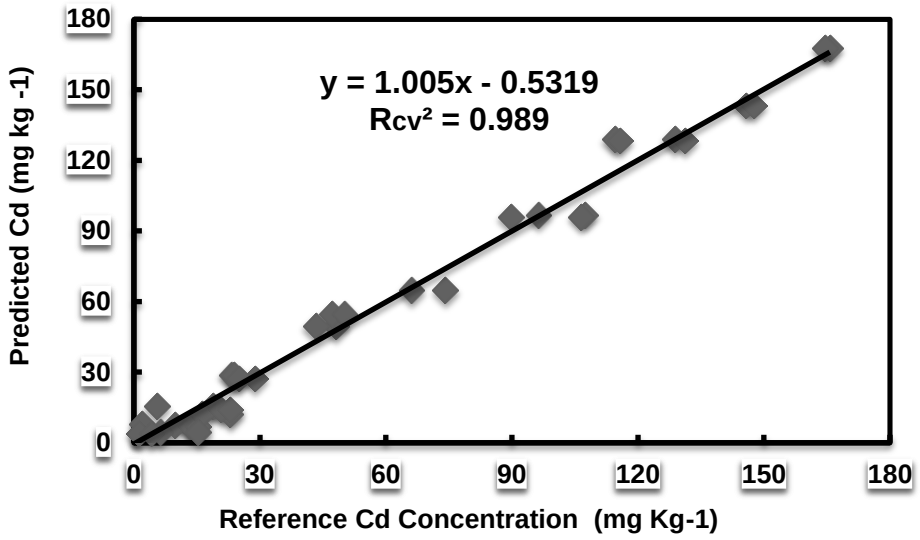


Figure 8.6 Cross validation: Partial Least Square Regression model based on leave one out of various levels of Cd concentration on 4 genotypes of tomato.

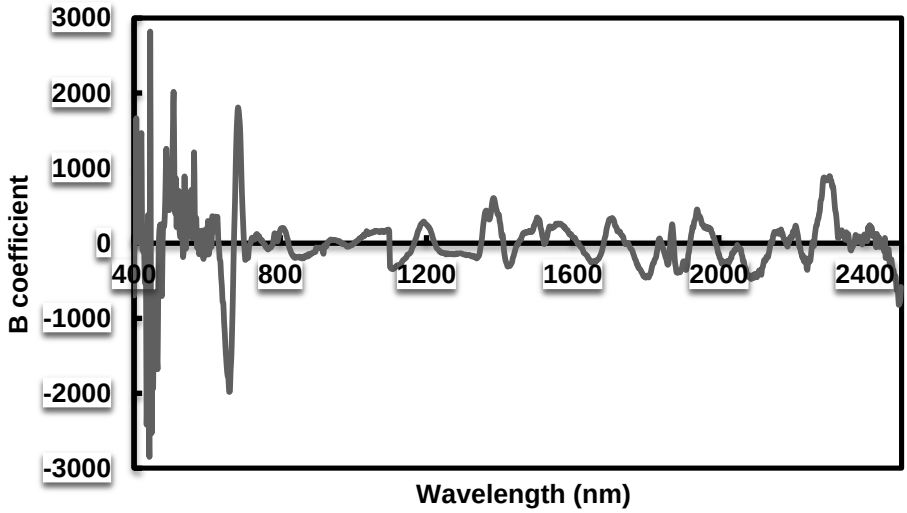


Figure 8.7 B-coefficient PLSR model of log 1/R spectra on various level of cadmium concentration on dried ground stems of 4 genotypes of tomato.

However, the spectral plot of regression coefficient log 1/R spectra data on Cd concentration indicated that there was no distinct signal on NIR region (800-2500 nm) except weak absorbance peak in 2300 nm region (Fig. 8.7). A strong peak was observed in visible region (636-710 nm) which indicates that there may be difference on chlorophyll content of the samples based on Cd concentration. As a result there should be an inverse relationship with chlorophyll and Cd concentration.

8.2.2.6 Calibration models

Various PLS calibration models have been calculated for the samples of the training set using different data pre-processing techniques and number of factors. Validation errors (RMSECV) of the final models for the different pre-processing methods are shown in Table 8.2 and 8.3 for PLSR calibration model of Cd concentration in the stem of tomato at the range 400-2500 nm. For all four tomato stem characteristics log 1/R spectra gave the lowest RMSECV value. Application of pre-processing methods for these 4 tomato stems characteristics reduce for most applied methods the required number of factors, but the RMSECV values become higher. Excellent results were obtained using log 1/R spectra for correlation coefficient of calibration (R_{cv}) and all statistical parameters. The differences in cross-validation error are relatively small. Normalisation, SNV and MSC show relatively higher RMSECV values than other characteristics. Kooistra *et al*. (2001) investigated that the possibilities of detecting Cd and Zn contamination levels in river flood plain using NIRS 6500 spectrophotometer in the range between 400 and 2400 nm. Various calibration models were calculated using different data pre-processing techniques. Validation results showed prediction errors (RMSEP) for the independent test set. Normalisation and MSC showed relatively higher RMSEP values for Cd and Zn levels.

Table 8.2 Calibration model performance of various spectral data pre-processing n=40

Pre-processing	Data type	Range (nm)	Mean	SD	R	RMSECV	Slope
ABS	ABS	400-2500	56.56	52.46	0.99	5.62	0.98
Mean Normalisation	ABS	400-2500	56.56	52.46	0.94	18.77	0.98
Derivatisation	d2A	400-2500	56.56	52.46	0.95	14.65	0.90
SNV	d2A	400-2500	56.56	52.46	0.95	15.92	0.88
MSC	d2A	400-2500	56.56	52.46	0.95	16.82	0.90
Baseline offset	d2A	400-2500	56.56	52.46	0.96	15.37	0.90
False reference	ABS	400-2500	56.56	52.46	0.97	13.02	0.94

ABS=absorbance spectra, SNV=standard normal variate, MSC=multiplicative scatter correction, d^2A=second derivative absorbance, R=correlation coefficient of calibration, SD=standard deviation, RMSECV=root mean square error of cross validation

Table 8.3 Prediction statistics A. Cross validation results for a group structure based on variety (i.e 4 groups), B. Cross validation result for a group structure based on Cd treatment, C1. Calibration result for sample 1-30 and 31-40, C2. Prediction result for model based on samples 1-30 in prediction of samples 31-40.

	Population	Mean	SD	R	RMSEP	SDR	Bias	Slope
A. 4 variety group Val set	40	56.56	52.46	0.47	46.69	1.13	2.01	0.31
B. 10 val. Group by Cd concentration	40	56.56	52.46	0.946	16.76	3.13	0.02	0.88
C1. Training set	Sample 1-30	57.46	53.5	0.958	15.34	3.49	-0.48	0.95
C2. Validation set	Sample 31-40	53.86	51.9	0.999	1.78	29.16	0.03	1
set 1-30	Val Set 31-40	53.86	51.9	-0.72	74.16	0.7	40.22	-0.24

SD=standard deviation, R= correlation coefficient of calibration, RMSEP=root mean square error of prediction, standard deviation ratio (SDR) = SD/RMSECV

In summary, near infrared spectroscopy based on effective wavelengths and the chemometrics software package was proposed to discriminate the powder form of dry stems of four tomato genotypes Big Beef, Tiny Tom, Burke's Backyard and Grosse Lisse. The calibration and validation results showed that NIRS was able to predict the Cd concentration in the powder form of dry stem of four tomatoes. The NIR predicted Cd concentration and the AAS measured Cd concentration are highly correlated. These results demonstrate that NIR technology can be used to predict cadmium concentration in plant tissues such as tomato stems.

As in the literature, encouraging calibration and cross validation results obtained using leave one out or groups based on Cd treatment level. However such approach lack independent test set. When independent set used- poor result. Likely use of full wavelength range and small sample number has lead to over fitting of the model. Previous studies should be re-examined with their addition of variety model with more than 50 samples.

8.3 Detection of tissue Cd concentration by XRF spectroscopy

Management of Cd contaminated soils and prevention of the use of contaminated plants require determination of Cd content of the plant, water or soil of concern. Conventional methods of determining metals in plants are based on wet digestion of dried samples and determining metals in the solution by atomic absorption spectrometry (AAS). This method is time-consuming and expensive (Batten 1998).

X-ray fluorescence (XRF) spectroscopy is one of the useful techniques as it is fast, non-destructive and economical, as it involves minimal sample preparation. Qualitative and quantitative analysis are accomplished without wet digestion and a large number of metals can be detected rapidly (Ene *et al*. 2010). Ene *et al*. (2010) have reported the use of XRF technique to determine contamination of soil with heavy metals such as arsenic (As), chromium (Cr), copper (Cu), nickel (Ni), palladium (Pb), vanadium (V) and zinc (Zn) in iron and steel industry in Romania. Concentrations of metals are high around the Iron and steel works factories and they decrease as one move away from these places. The large variation was found in the quantities of V and Zn found in these areas. The concentrations of As, Pb, Zn, Cu, Cr, V and Ni were higher than those found in natural. Cosio *et al*. (2005) determined Cd concentrations in the shoots of two ecotypes of *Thlaspi caerulenscens,* Ganges and Prayon using electron microscopy coupled with energy dispersive X-ray microanalysis. The Prayon ecotype grew better than Ganges at 50 µM Cd. Ganges was more affected by low and medium Cd levels (5 and 10 µM Cd). Radulescu *et al*. (2010) compared the analytical possibilities of energy dispersive X-ray spectrometry and Flame Atomic Absorption spectrometry (FAAS) methods to quantify heavy metal transfer from soil to mushrooms. The concentrations of Cd, Cr, Ni, Pb, Co and

Ti, in the fruiting body of toxic mushrooms were higher than those in non-toxic species. The highest Cd concentration was found in the two toxic species *Hypholoma fasciculare* (0.35 mg/kg/DW) and *Amanita phalloides* (0.30 mg/kg/DW).

The aim of this experiment is to test if the X-ray fluorescence spectroscopy can be used to detect Cd concentrations in dried tomato tissues, via comparing the results of wet digestion method with those of XRF.

8.3.1 Materials and Methods

8.3.1.1 Establishment of tomato plants in sand culture

Washed river sand was procured from a local landscape supplier. The sand was dried in a dryer at 60˚C for two days. Dried sand was filled into each pot and weighed (7 kg). The pots were placed on a glasshouse bench that was lined with a thick plastic sheet to prevent Cd solution leaking from the pots. Saucers were placed under the pots to ensure that excess water was collected in the saucer and was allowed to be reabsorbed by the plant. Cadmium stock solution (100 mM) was prepared as described in Chapter 3.6. Ten seeds of each genotype were sown in each pot. In each pot 200 ml of reverse osmosis (RO) water was poured until the seeds were germinated. After 7 days of germination, three Cd treatments, viz. 0, 100, 500 µM Cd were imposed using three replications. Preparation of hydroponics nutrient solution with or without Cd is described in Chapter 3.7. After seed germination, Manutec (Part 1=N.P.K.7.6/3.1/18.2 + Part 2; Trace elements=calcium nitrate) hydroponics nutrient solution with or without Cd was added to all pots at 200 ml/pot during the first week and the second week. The experiment was run in a glasshouse which was maintained at a temperature of 25-32°C using an evaporative cooler. The experiment was replicated twice.

8.3.1.2 Tissue analysis for cadmium- Atomic absorption spectroscopy

The dried tissues were ground into fine powder (<1.5 mm) using a Culatti hammer mill (MFC Mikro-Feinmuhle-Culatti). The powder (0.4 g) was placed in 10 ml plastic tube (PPTR) and 2 ml of nitric acid (HNO_3) was added along with a drop of 30% hydrogen peroxide (H_2O_2). The plastic tubes were placed in the fume hood and were left to digest over night. On the following morning, 3 ml of Milli-Q water was added and then placed in a water bath (Grant Instruments, Cambridge Ltd) at 70ºC for 4 hours. After digestion, the volume of the solution in the tube was made up to 10 ml using Milli-Q water. The tube was centrifuged at 15,000 g for 15 minutes. The Cd concentrations of the supernatant were determined using AAS spectrophotometer (Varian Australia Pty. Ltd).

8.3.1.3 Tissue analysis-X-ray fluorescence spectroscopy (XRF)

The XRF was set up on a laboratory bench to facilitate sample measurement. The ground plant sample was filled into the XRF plastic cup to a depth of 6 mm. A disc of filter paper was placed followed by a piece of batting. The batting prevented the sample from moving during loading. The sample was analysed using X-ray fluorescence spectrometer (Niton XL 3t 950 GOLDD+) in “mining Cu/Zn mode” at the settings of (30 s main, 30 s low, 30 s high and 30 s light).

8.3.1.4 Statistical analysis of data

Tissues Cd concentrations from wet digestion and from XRF spectroscopy were plotted and the correlation coefficient of determination (R^2) was calculated using Microsoft Excel. Since the Cd concentrations obtained from AAS correlated very well with those obtained from XRF, it was presumed that the XRF would provide

similar prediction for other elements such as cadmium (Cd), calcium (Ca), palladium (Pd), zinc (Zn), silicon (Si), chlorine (Cl), sulphur (S), molybdenum (Mo), niobium (Nb), zirconium (Zr), rubidium (Rb), potassium (K) and phosphorus (P), and hence the Cd concentrations of the tissues used in this study were correlated with those of other elements with the view to testing if the uptake of Cd will interfere with the uptake of other elements.

8.3.2 Results

Testing the XRF for accuracy

The factory supplied standard samples were analysed using X-ray fluorescence spectrometer in "mining mode" at the settings of 30 s main, 30 s low, 30 s high and 30 s light.

Several standard samples that were provided by the XRF manufacturer were scanned under the same conditions along with the tomato samples. The measured readings from these samples were correlated with those recorded by the XRF.

As shown in Figures 8.8A and 8.8B, the tissue Cd concentrations derived from both techniques (AAS vs. XRF) correlated well (R^2=0.98) at both wider and narrow ranges of tissue Cd concentration. This indicated that the use of XRF is reliable for determining tissue Cd concentrations in tomato.

Wet digestion is time consuming and is expensive. The current trial clearly shows that the XRF can be used to determine Cd concentrations in tomato plants. Makinen *et al*. (2005) also demonstrated the reliability of XRF in detecting As, Cu and Cr elements are compared with using portable field X-ray fluorescence (XRF)-analyzer X-MET 2000 and flame atomic absorption spectrometry (FAAS) to analyse As, Cu

and Cr in species. Analysis with XRF and FAAS results showed excellent correlation with As and Cu. However, they reported poor correlations between the wet digestion method and XRF analysis for Cr suggesting that the XRF technology is more reliable for some elements than the others (Makinen 2005).

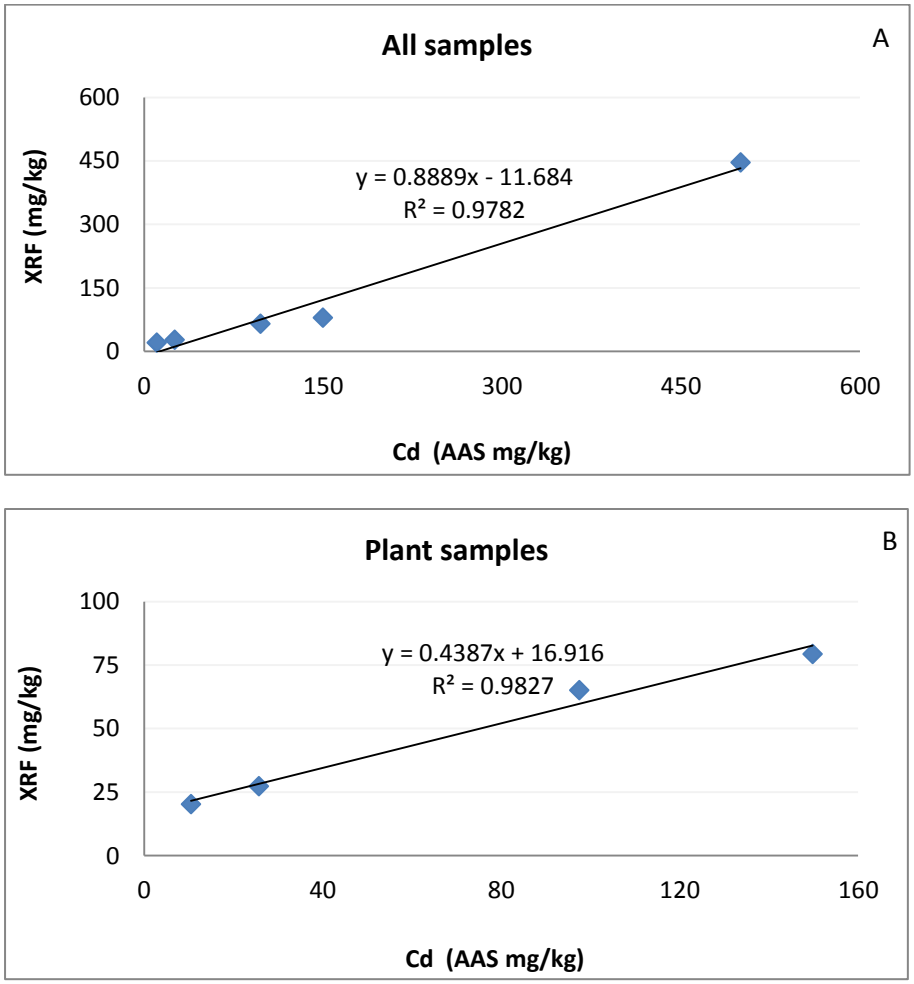


Figure 8.8 Relationship between the measured readings and the nominated readings of standard plant samples at (A) wider range of Cd concentrations and at (B) narrow range of Cd concentrations.

Thirty one ground stem samples of tomato genotypes Grosse Lisse and Big Beef were analysed for Cd using wet digestion method (see Section 8.3.1.2) and via XRF (8.3.1.3). The Cd concentrations of AAS method correlated well (R^2=0.98; Fig. 8.9) with those of XRF method in detecting Cd concentrations in plant tissues.

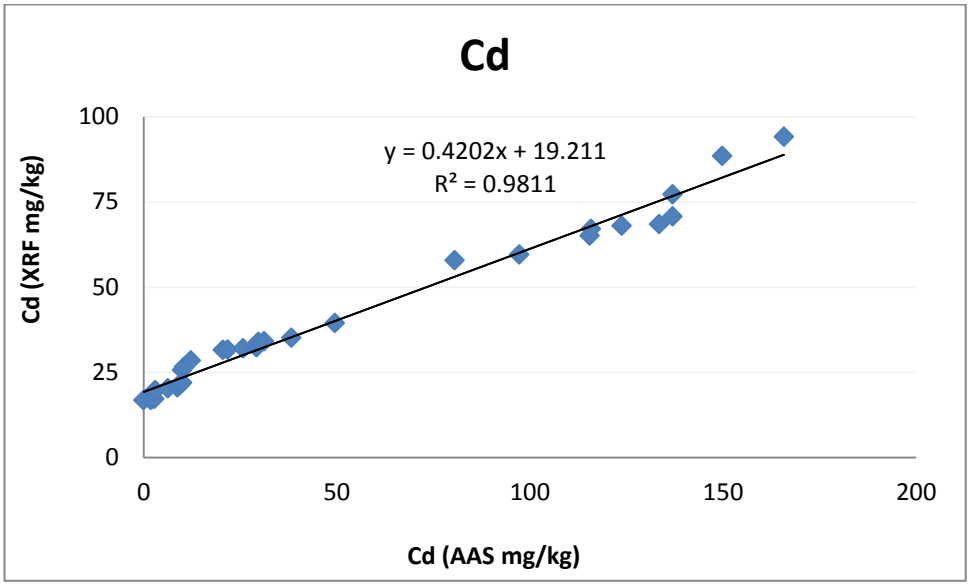
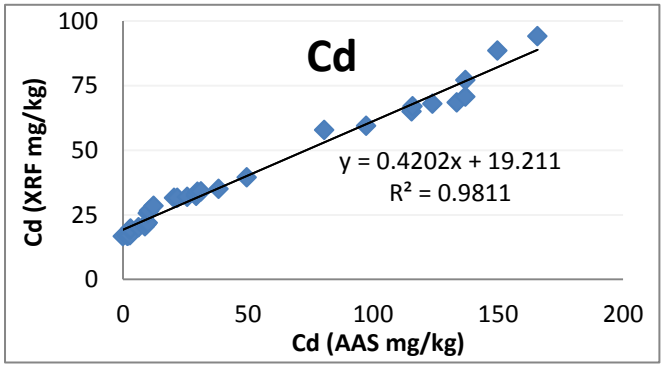


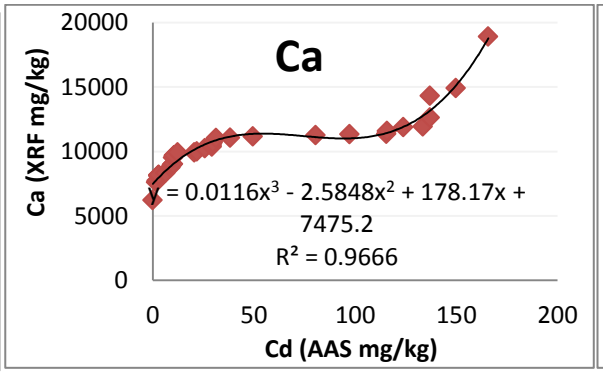
Figure 8.9 Relationship between X-ray fluorescence measured Cd concentration and AAS measured Cd concentration in the stem tissues of two tomato genotypes.

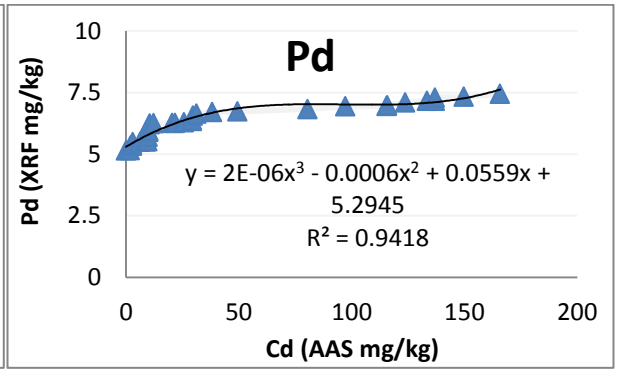
Since the XRF Cd readings correlated well with those derived from AAS (wet digestion), it was presumed that the XRF would provide similar prediction for other elements. Accordingly, the Cd concentrations of the 31 tomato samples were correlated with the concentrations of other elements such as Ca, Pd, Zn, Si, Cl, S, Mo, Nb, Zr, Rb, K and P (Fig. 8.10). The XRF Cd concentrations increased sharply as the tissue Cd concentration increased up to 150 mg/kg. The XRF Ca concentration had 3 types of responses. Calcium (Ca) concentration increased as the Cd increased up to 50 mg/kg; then it did not change up to 120 mg/kg and then it increased very fast. The XRF palladium (Pd) concentration increased up to 50 mg/kg and then it plateau. The XRF measured Zinc (Zn) followed similar pattern as calcium. Silicon (Si) concentration increased, the increment was kept steadily as the tissue Cd concentration increased but at a slow pace. The tissue chlorine (Cl) concentration increased rapidly as the tissue Cd concentration increased; up to 50 mg/kg and then it accumulated slowly. The XRF detected sulphur (S), Rubidium (Rb), potassium (K) and phosphorus (P) all increased in three steps; very fast, very slow and show

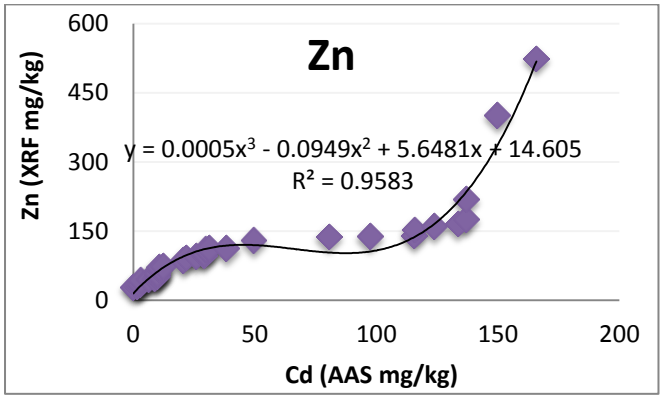
increment. The XRF determined Zirconium (Zr) concentrations showed similar patterns as Molybdenum (Mo) and Si, with respect to Cd. The XRF detected Niobium (Nb) uptake was similar to that of palladium (Pd). The XRF detected Rubidium (Rb) and Phosphorus (P) and sulphur (S) had similar patterns with patterns of increment in relation to Cd.

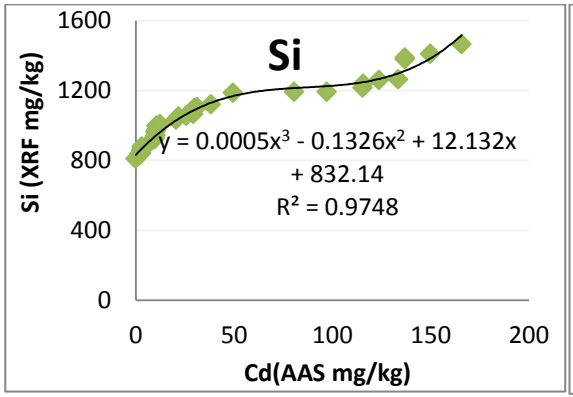
These results show that the accumulation of Cd in the stem tissues of tomato will also enhance the accumulation of Ca, Zn, S and K. The Cd accumulation had only slightly enhanced the uptake of Mo and Zr, but had little effect on the accumulation of Pd, Nb and Mo in the stem tissues of tomato.

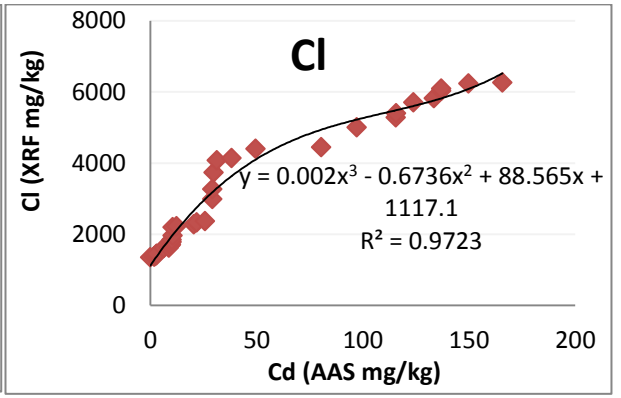

Cd
y = 0.4202x + 19.211
R² = 0.9811
Cd (XRF mg/kg)
Cd (AAS mg/kg)
0
25
50
75
100
0
50
100
150
200

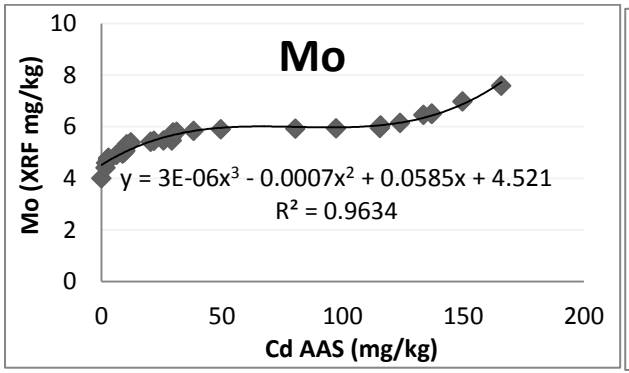

Ca
y = 0.0116x³ - 2.5848x² + 178.17x + 7475.2
R² = 0.9666
Ca (XRF mg/kg)
Cd (AAS mg/kg)
0
5000
10000
15000
20000
0
50
100
150
200

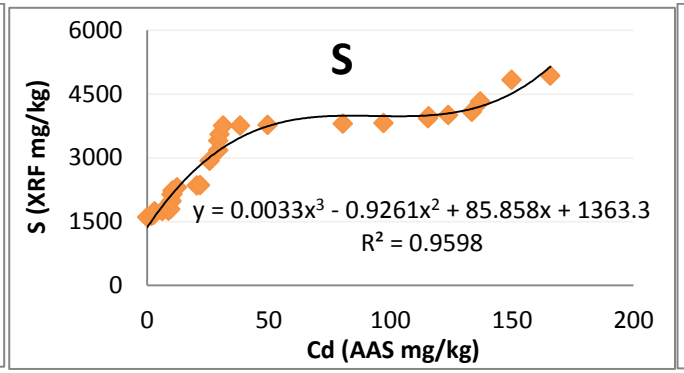

Pd
y = 2E-06x³ - 0.0006x² + 0.0559x + 5.2945
R² = 0.9418
Pd (XRF mg/kg)
Cd (AAS mg/kg)
0
2.5
5
7.5
10
0
50
100
150
200

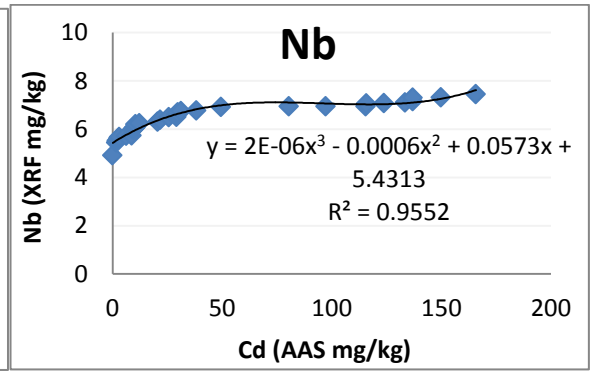

Zn
y = 0.0005x³ - 0.0949x² + 5.6481x + 14.605
R² = 0.9583
Zn (XRF mg/kg)
Cd (AAS mg/kg)
0
150
300
450
600
0
50
100
150
200

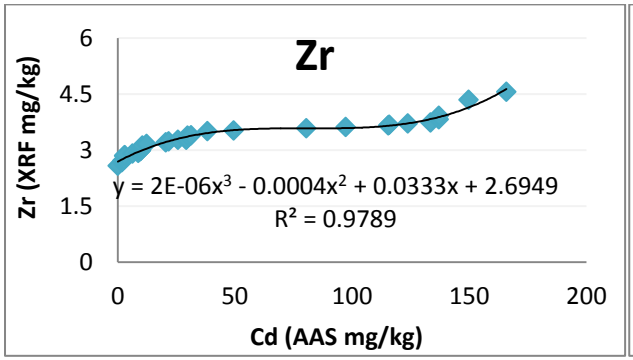

Si
y = 0.0005x³ - 0.1326x² + 12.132x + 832.14
R² = 0.9748
Si (XRF mg/kg)
Cd(AAS mg/kg)
0
400
800
1200
1600
0
50
100
150
200

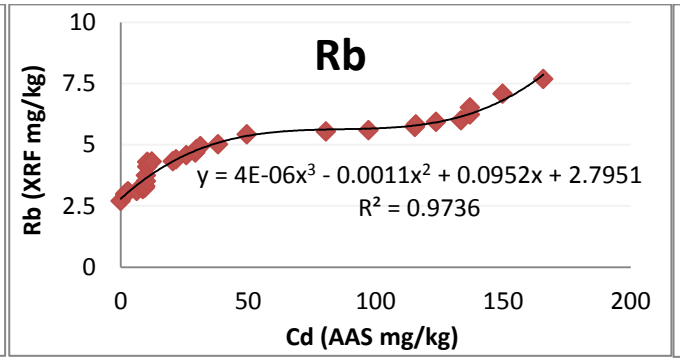

Cl
y = 0.002x³ - 0.6736x² + 88.565x + 1117.1
R² = 0.9723
Cl (XRF mg/kg)
Cd (AAS mg/kg)
0
2000
4000
6000
8000
0
50
100
150
200

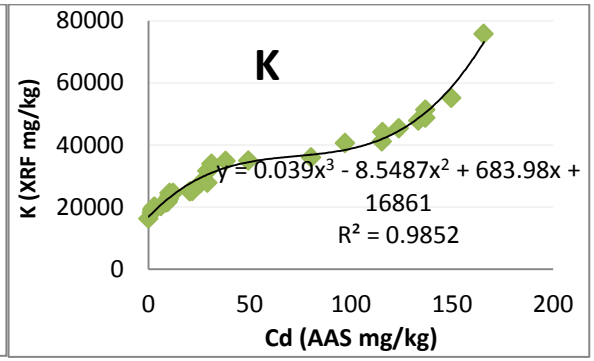

Mo
Mo (XRF mg/kg)
Cd AAS (mg/kg)
y = 3E-06x³ - 0.0007x² + 0.0585x + 4.521
R² = 0.9634


S
S (XRF mg/kg)
Cd (AAS mg/kg)
y = 0.0033x³ - 0.9261x² + 85.858x + 1363.3
R² = 0.9598


Nb
Nb (XRF mg/kg)
Cd (AAS mg/kg)
y = 2E-06x³ - 0.0006x² + 0.0573x + 5.4313
R² = 0.9552


Zr
Zr (XRF mg/kg)
Cd (AAS mg/kg)
y = 2E-06x³ - 0.0004x² + 0.0333x + 2.6949
R² = 0.9789


Rb
Rb (XRF mg/kg)
Cd (AAS mg/kg)
y = 4E-06x³ - 0.0011x² + 0.0952x + 2.7951
R² = 0.9736


K
K (XRF mg/kg)
Cd (AAS mg/kg)
y = 0.039x³ - 8.5487x² + 683.98x + 16861
R² = 0.9852

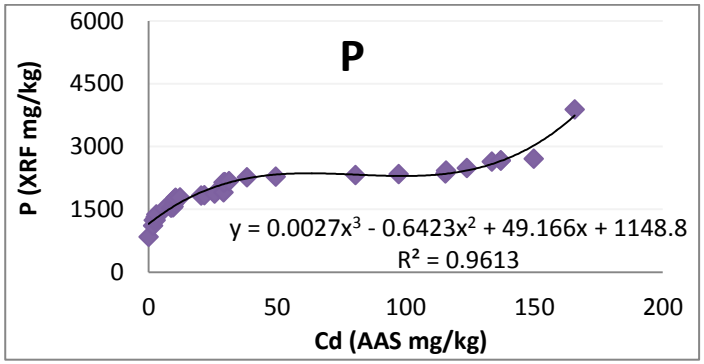


Figure 8.10 Relationship between AAS determined Cd and XRF determined Cd, Ca, Pd, Zn, Si, Cl, S, Mo, Nb, Zr, Rb, K and P.

8.4 Discussion

In this study, X-ray fluorescence analysis was applied to determine cadmium concentration of ground tomato stems. Two genotypes viz. Grosse Lisse and Big Beef were used and the plants were grown in sand culture, treated with Cd and were harvested at 7 weeks and 17 weeks after sowing. Tissues Cd concentration was determined by AAS and XRF spectroscopy. The XRF reading was taken in "mining Cu/Zn mode".

A high correlation R^2= 0.98 was found between AAS and XRF readings (Fig. 8.9) indicating that the XRF can be used in detecting Cd concentrations in plant tissues. Yamaguchi *et al*. (2011) used synchrotron micro X-ray fluorescence spectrometry to determine Cd in two species of egg plant *Solanum melongena* contained higher concentrations of Cd than *S. torvum* when they were treated with 1.33 µM $CdCl_2$. Margui *et al*. (2006) assessed metal availability to *Betula pendula* at a Pb-Zn mining factory, and showed that Pb and Zn accumulation was negatively correlated with K, and positively correlated with Ca. Lead and Zn accumulation were also negatively correlated and also were the Zn and Mn in *Betula pendula*. Khuder *et al*. (2009) assessed Syrian medicinal plant samples (*Anisum vulgare, Glycyrrhiza glabra and Artemisia herba-alba*) for mineral elements using TXRF and XRF methods. The highest concentration was noted for Ca and K and the lowest concentration was found for Br and Rb in *Artemisia herba-alba*. Anjos *et al*. (2002) used XRF to determine K, Ca, Ti, Mn, Fe, Cu, Zn, Br, Rb, Sr, Zr and Pb concentrations in the roots and leaves of radish. The results indicated that K and Pb concentrations were higher in the root tissues of radish, whereas the concentrations of Ca, Ti, Mn, Fe, Cu,

Zn, Br, Sr and Zr were higher in the leaves. Mino and Yukita (2005) determined bromine content of different vegetables using X-ray fluorescence spectrometry. The results showed high bromine content (more than 1000 ppm) in Chinese cabbage, Shungiku and Komatsuna. Xin *et al*. (2009) studied distribution of mineral elements in the roots and leaves of greenhouse-raised spinach using synchrotron radiation XRF. They found that the relative concentrations of K, Ca, Mn, Fe, Co, Ni, Cu and Zn were higher in spinach leaves and roots but the K content in the root was slightly lower.

Increased uptake of one element could either suppress the uptake of other elements or it could enhance the uptake and accumulation of another elements. In this study, Cd uptake was related to the uptake of other elements such as Ca, K, Cl, S, Zn, Rb and P. The results showed that Cd did not inhibit the uptake of any of the above elements (Fig 8.10). However, increase in Cd also highly increased the accumulation of S, K, Rb. Various other researcher have reported increased, decreased or neutral effect on the uptake of one element with respect to uptake of other elements (Aswathappa & Bachelard 1986).

Overall, this study suggests that XRF spectroscopy provides reliable data for determining cadmium concentrations in plant tissues, and the increased uptake of Cd would also increase the uptake of other elements. Accumulation of cadmium in the stem tissue of tomato does not seem to inhibit the uptake and accumulation of many of the tested elements.

Chapter 9

General Discussion

Tomato is one of the most important vegetable crops both for commercial use and for its utilisation in scientific studies such as tissue culture and genetic transformation. It is grown in the field, glasshouses and net houses. Its production from semiurban areas may lead to accumulation of Cd in tomato, which will eventually enter into the food cycle. Removal of Cd from semiurban areas via phytoremediation may reduce exposure of humans to Cd. This study explored the use of cultivated and wild genotypes of tomato as Cd accumulators with the view to using them in phytoremediation of Cd contaminated sites.

The presence of non essential heavy metals in the air, soil and water can cause environmental pollution and phytotoxicity to plants. Cadmium is a non essential element for plant growth, and is released into the environment from power stations, heating systems, electrolysis plants, industry and from smelting ore by mining industries.

The micropropagation method can regenerate large number of plants which can be used to remediate metal polluted sites. Phytoremediation technique is the use of metal hyperaccumulating plants for cleanup of metals from metal polluted soils.

The aim of the current study was to select suitable genotypes of tomato that will tolerate high concentrations of cadmium in the substrate, and accumulate large quantities of Cd in their shoots with the view to using them in phytoremediation of Cd contaminated sites.

9.1 Optimising growth conditions for establishing tomato in tissue culture

The first experiment (Chapter 4) was conducted to test seed germination and to optimise growth of plants in tissue culture, including response of tomato to shoot multiplication procedures from hypocotyls, cotyledons and shoot tips using Murashige and Skoog (MS) basal medium. The highest seed germination (100%) was observed in 7 of the 15 genotypes. More than 80% seed germination was found in 7 of the 15 genotypes. These results are consistent with those reported for Okra (*Abelmoschus esculentus*) in MS basal medium where 80% of the seeds germinated (Ganesan *et al.* 2007). Similarly tomato cultivar Omdurman produced 96% seed germination in full strength MS medium and 100% seed germination in half-strength MS medium (Ishag *et al*. 2009).

The numbers of shoots arising from hypocotyls were higher than those from cotyledons and shoot tips in all genotypes except in the Heir Loom and Tiny Tom. Callus formation was recorded in cotyledons and shoot tips of all genotypes. No callus formation was found in the hypocotyls of 9 genotypes. Jabeen *et al*. (2005) reported production of higher number of shoots from hypocotyls and shoot tips of tomato cultivar Riograndea in MS medium containing growth regulators compared to tomato cultivar Roma, Money maker, Nagina and Feston. Callus formation from shoot tip increased in Turkish cowpea cv. Akkiz supplemented with MS basal medium and 0.5 mg/l benzylaminopurine (Aasim *et al*. 2008).

The results indicated that hypocotyls were the best explants for shoot induction in tomato. Based on this experiment, we selected three genotypes (Burke's Backyard, Red Cherry and San Marzano-2) for the second and third experiments.

The second experiment (Chapter 4) was conducted in tissue culture using three genotypes of tomato. Seed germination percentage, shoot height, fresh shoot weight and dry shoot weight were determined for plants grown in MS media and sown with 3, 6, 9 and 12 seeds using five replications. Highest numbers of seedlings were produced in treatments sown with 12 seeds.

Heights of the germinated seedlings revealed the presence of tallest seedlings in treatments sown with 3 seeds per tube. The results showed that the treatment with 12 seeds produced better germination percentage, but that with 3 seeds produced robust and healthy seedlings. Based on these experiments, all further experiments were conducted using 3 seeds per tube.

The third experiment (Chapter 4) was conducted using three genotypes of tomato treated with five different media (agar, wiped slant agar, unwiped slant agar, potting and sand media) for 5 weeks. In this experiment, more than 78% seed germination was observed in three genotypes (Burke's Backyard, Red Cherry and San Marzano-2) that were treated differently in terms of the media composition and preparation of the media.

Amongst the three media used, plants grown in agar media produced the highest dry weight but the shoot height was low compared to those grown in sand and potting media.

The shoot dry weight of three genotypes was higher than root dry weight in the three media. The agar medium produced the highest shoot to root ratio in contrast to sand media which produced the lowest shoot to root ratio. This experiment clearly shows

that the agar media is better suited for shoot growth, and the potting media is good for a balance growth of roots and shoots.

The wiped and unwiped slant agar experiment was conducted to minimise condensation in the tubes which would otherwise increase the chances of contamination. The results showed that the wiped tubes had less contamination than unwiped tubes. This experiment suggested that the contamination in tissue culture can be minimised by reducing condensation in large tubes, and wiping tubes can help reduce this contamination.

9.2 Cadmium tolerance of 25 genotypes of tomato

The fourth experiment (Chapter 5) was conducted using 25 genotypes of tomato with the view to identifying the genotype that grew well and transported most Cd into their shoots. Each of the 25 genotypes was grown in MS media containing seven concentrations (0, 10, 30, 100, 200, 500 and 1000 µM) of Cd. The plants were harvested after 5 weeks of growth in tissue culture tubes. A hundred percent seed germination was found in 10 of 25 genotypes. These results are consistent with those reported by Ishag *et al*. (2009) who found 96% seed germination in full strength MS medium and 100% seed germination in half-strength MS medium in tomato cultivar Omdurman. Salvatore *et al*. (2008) reported that more than 90% of seed germination was found in lettuce, broccoli, tomato and radish in media containing 0-1024 µM Cd.

The shoot height and shoot weight were higher in treatments containing low concentrations of Cd and they were low at higher levels of Cd in all genotypes. Shoot height of tomato cv. Seokwang decreased when treated with 50 and 100 µM

Cd (Cho 2004). Leaf dry weight of tomato cv. Micro-Tom decreased at 200, 500 and 1000 µM $CdCl_2$ after 20 days of growth (Grato *et al.* 2008).

The tomato genotypes produced increased shoot dry weight at 10, 30, 100 and 200 µM Cd but the dry weights declined at 500 and 1000 µM Cd. Our aim was to identify the genotype that grew well at high levels of Cd and also accumulated most Cd.

The 14 of 25 genotypes were highly sensitive to the highest Cd concentration 1000 µM Cd. Seedlings did not grow well and they were crawling on the media. The highest shoot Cd concentration was observed in Nash2 and Grosse Lisse and the lowest was found in Big Beef treated with 1000 µM Cd after 5 weeks of exposure. Therefore, genotype Nash2 and Grosse Lisse can be used in phytoremediation. Grato *et al.* (2008) reported more than 50% reduction in plant leaf growth in tomato cv. Micro Tom treated with 500 and 1000 µM $CdCl_2$ as compared to control after 75 days of exposure.

The leaf Cd uptake rate was calculated for the 25 genotypes using the leaf weight and leaf Cd concentration. The low leaf Cd uptake (< 10 µg/plant) was observed in 6 genotypes, the medium Cd uptake (10- 20 µg/plant) was found in 12 genotypes and the high Cd uptake (20-35 µg/plant) was found in 6 genotypes. Yang *et al.* (2004) reported that cadmium uptake from the shoots of *Sedum alfredii* increased at 400 µM and it decreased at 800µM due to severe reduction in shoot weight at higher Cd concentration (800 µM Cd).

The results of this study showed that 15 of the 25 tomato genotypes were tolerant to high Cd toxicity for seed germination (100% seed germination). However, after seed germination, 15 of the 25 tomato seedlings were hypersensitive to high Cd

concentrations (1000 µM). All 25 tomato genotypes were tolerant to low levels (10 and 100 µM) as their shoot weight did not differ from those of the control, but were sensitive high Cd levels 500 and 1000 µM. The ten of the 25 genotypes were tolerant and accumulated the high Cd level 500 µM in their shoots. Overall the highest Cd uptake was found in CLt91t6D4 (32.4 µg/plant).

Based on this experiment, 4 genotypes were selected for further investigation in the glasshouse.

9.3 Physiology of cadmium tolerance in four genotypes of tomato that differ in cadmium accumulation

The fifth experiment (Chapter 6) was conducted using four genotypes of tomato (Big Beef, Tiny Tom, Burke's Backyard and Grosse Lisse). These genotypes were grown in sand culture in a glasshouse and were treated with five concentrations of Cd (0, 30, 200, 500 and 1000 µM). Shoot height and shoot weight were determined at three weeks, five weeks, seven weeks and nine weeks after exposing the plants to Cd in a glasshouse. More than 97% seed germination was observed in the 4 genotypes demonstrating that Cd did not have any adverse effect on seed germination. In contrast to these results, Meng *et al*. (2009) reported sensitivity of rape oilseed (*Brassica* napus) to cadmium 200 and 400 µM of Cd.

The shoot height was not affected by Cd in all 4 genotypes. This again was contradictory to that reported for *Sedum alfredii* which showed a reduction in shoot height from 200 to 800 µM Cd (Yang *et al.* 2004).

In 30 µM Cd, the leaf and stem dry weight of Grosse Lisee were two times higher than the control after 3 weeks of exposure to Cd. The leaf dry weight decreased in Big Beef and Tiny Tom at 1000 µM Cd compared to control after 5 weeks of

exposure. The leaf dry weight decreased in Burke's Backyard at 1000 µM Cd compared to control after 7 weeks. The leaf dry weight decreased in Tiny Tom at 1000 µM Cd but increased in Grosse Lisse at 30 and 500 µM when compared to control after 7 weeks of exposure to Cd. The shoot dry weight of *Sedum alfredii* decreased at 800 µM Cd compared to control (Yang *et al*. 2004). The leaf dry weight of Arum was not different between the treatments at 10 and 50 µM Cd and the shoot dry weight of Radish was also not different between the treatments at 1.5, 2.5, 5 and 10 µM Cd but the leaf dry weight of Water spinach reduced at 10 µM Cd as compared to control (Kashem *et al.* 2008).

The stem dry weight decreased in Big Beef at 1000 µm Cd after 3 weeks, 5 weeks and not different at 7 weeks but increased at 200 and 500 µm after 7 weeks when compared to control. The stem dry weight decreased in Tiny Tom at 1000 µm Cd after 3, 5, 7 and 9 weeks of exposure compared to control. The stem dry weight decreased in Burke's Backyard at 1000 µm Cd after 3 weeks and 7 weeks but increased at 200 µm after 7 weeks compared to control. The stem dry weight increased in Grosse Lisse at 30 µm Cd after 3 weeks, at 500 µm after 7weeks and at 1000 µm Cd after 9 weeks. The stem dry weight decreased in arum at 50 µM Cd but not difference at 10 µM Cd as compared to control. In radish and water spinach treated with 10 µM Cd decreased the stem dry weight but increased at 2.5 µM Cd as compared to control (Kashem *et al*. 2008). The stem dry weight decreased in tomato at 100 µm Cd when compared to control (Lopez- Millan *et al*. 2009).

The leaf Cd concentration in Big Beef was higher than that in Tiny Tom, Burke's Backyard and Grosse Lisse at 1000 µM Cd after 3 and 7 weeks exposure but it was

lower than that in Burke's Backyard and Grosse Lisse at 1000 µM after 5 weeks of exposure.

The highest stem Cd concentration and Cd uptake was observed in Big Beef at 1000 µM Cd after 7 weeks of exposure.

Large variations in Cd uptake and tissue Cd concentrations have been reported in literature. For example, Cd concentration increased in the leaves and stems of *Sedum alfredii* treated with 400 µM Cd compared to control (Yang *et al*. 2004). Incontrast, Boominathan and Doran (2003) reported a reduction in Cd in *Thlaspi caerulescens* at 178 µM Cd than at 100 µM Cd.

The leaf Cd uptake increased in Big Beef and Burke's backyard at 500 µM after 3 weeks. In Grosse Lisse, Burke's Backyard and Big Beef, Cd concentration increased at 1000 µM Cd after 5 and 9 weeks. The leaf Cd uptake higher in Big Beef and Burke's Backyard than Grosse Lisse and Tiny Tom at 1000 µM after 7 weeks exposure.

Higher shoot Cd concentration and Cd uptake was found in Big Beef when compared to Tiny Tom, Burke's Backyard and Grosse Lisse at 1000 µM Cd after 7 weeks of exposure. The leaf cadmium uptake of shoots and roots linearly increased in *Sedum alfredii* up to 400 µM Cd but decreased at 800 µM Cd (Yang *et al*. 2004). The shoot Cd uptake increased in pea at 30 µM Cd compared to 1 and 3 µM Cd (Lima *et al*. 2006).

The results showed that the Cd toxicity did not affect seed germination in four tomato genotypes. The plant height of Grosse Lisse was not affected by high Cd concentration (1000 µM) unlike that in Tiny Tom. The leaf dry weight was higher

than stem dry weight in all genotypes Big Beef, Grosse Lisse, Burke's Backyard and Tiny Tom after 3, 5, 7 and 9 weeks. The higher Cd concentration and uptake was found in Big Beef after 3weeks and 5 weeks and in Grosse Lisse after 5 weeks and 9 weeks. Big Beef, Grosse Lisse and Burke's Backyard could therefore be used in the phytoremediation of cadmium polluted soil that contain maximum concentration of 500 and 1000 µM Cd.

These results indicated that the relative response of genotypes in tissue culture experiment was similar to that observed in sand culture in the glasshouse. However, the leaf Cd concentrations was up to 4 times higher in *in vitro* cultured plants than those grown in glasshouse in sand culture. This may possibly be due to increased availability of Cd in the tissue culture media.

9.4 Comparison between two tomato genotypes that differ in cadmium uptake, accumulation and distribution in their shoots

The sixth experiment (Chapter 7) was conducted using two genotypes Big Beef and Grosse Lisse to determine their longer term responses to Cd in a glasshouse. Seedlings of the above genotypes were exposed to 0, 100 and 500 µM Cd in a glasshouse in sand culture for 17 weeks. More than 93% seed germination was observed in the two genotypes, showing little effect of Cd on seed germination of tomato. In contrast, Munzuroglu and Zengin (2006) reported that the barley seed germination decreased with increasing Cd concentration and were found to be 39%, 66%, 87% and 97% for Cd concentration of 2500 µM Cd, 4500 µM Cd, 6500 µM Cd and 8500 µM Cd respectively when compared to control.

The Cd did not affect shoot height of Grosse Lisse but increased in Big Beef at 100 and 500 µM Cd compared to control. The response recorded for *Sedum alfredii* wherein the plant height decreased at 200 to 800 µM Cd as compared to control (Yang *et al.* 2004).

The leaf dry weight of the two genotypes was higher than stem dry weight in 2 genotypes after 17 weeks of exposure to Cd. The leaf and stem dry weight of genotype Big Beef and Grosse Lisse tolerant the effects of Cd treated with 100 µM and 500 µM after 17 weeks of exposure. The leaf and stem dry weight decreased in Big Beef at 500 µM Cd after 7 weeks when compared to control. Lopez-Millan *et al*. (2009) found that, the leaf and stem dry weight decreased in tomato cv. Tres Cantos when treated with 100 µM Cd as compared with the control.

Chlorophyll content was not different between all Cd treatments. Cherian *et al*. (2007) showed that the chlorophyll content decreased in tomato (*Lycopersicon esculentum*) treated with 7120 µM Cd but increased at 1780 µM Cd as compared to the control.

The leaf Cd concentration was higher in Grosse Lisse than in Big Beef at 500 µM Cd after 17 weeks of exposure. The stem Cd concentration was not different between the two genotypes after 17 weeks. The response of shoot cadmium contents increased sharply at 50 µM $CdCl_2$ in three genotypes of lettuce compared to control (Zorrig *et al*. 2010).

The leaf Cd uptake was two times higher in Grosse Lisse than Big Beef at 500 µM Cd after 7 and 17 weeks of exposure to Cd. The stem uptake increased up to 4 times higher in Grosse Lisse than Big Beef at 500 µM Cd after 7 weeks of exposure to Cd. The stem Cd uptake significantly increased in both genotypes at 500 µM Cd

compared to 100 µM Cd after 17 weeks of exposure. The cadmium uptake increased in root, shoot and grain of pea at 214 µM Cd and the uptake of chromium (Cr) and copper (Cu) increased in root, shoot and grain of pea at 2616 µM Cr and 21056 µM Cu respectively when compared to control (Wani *et al.* 2008).

The results showed Grosse Lisse had higher leaf Cd concentrations and Cd uptake than Big Beef after 17 weeks.

9.5 Testing the use of Near-Infrared Reflectance (NIR) and X-ray fluorescence (XRF) spectroscopy to detect cadmium in tomato tissues

The seventh experiment showed in Chapter 8. Near-infrared reflectance spectroscopy method is a rapid, reliable, non-destructive and user friendly method and is useful for qualitative and quantitative analysis of agricultural product for heavy metals. This experiment was conducted using near infrared reflectance spectrophotometer and was applied to determine the cadmium content levels of four tomato stems using Unscrambler chemometrics statistical software. Twenty dried stem samples were scanned in the NIR System model 6500 visible NIR spectrophotometer in the reflectance mode over a wavelength range from 400 to 2500 nm. The results showed predicted and measured cadmium concentrations are highly correlated. The correlation coefficient of determination R^2=0.9909 was observed. There was no obvious relationship between log 1/R of d^2 log 1/R spectra of Cd level in the sample. A low RMSECV was achieved with a start around 500 nm and a finish up to 1350, or 1850 or 2300, or a start at around 950 and a finish around 1300, or a start around 2100 and a finish around 2300 nm. PC1 and PC2, derived from log 1/R or d^2 log 1/R of the samples. Two separated cluster PC1 and PC2 are observed. For all four tomato stem characteristics log 1/R spectra gave the lowest RMSECV value. Application of

pre-processing methods for these 4 tomato stems characteristics reduce for most applied methods the required number of factors, but the RMSECV values become higher.

Encouraging calibration and cross validation results obtained using leave one out based on Cd treatment level. However, such approach lack independent test set. When independent set used- poor result. Likely use of full wavelength range and small sample number has lead to over fitting of the model. Previous studies should be re-examined with their addition of variety model with more indipendent samples.

A successful calibration was developed between the NIR spectra of filters and the amount of cadmium on the filters in lake picoplankton by using NIR system 6500 and wavelength range from 400 to 2500 nm. The correlation coefficient of determination R^2 value is 0.921 (Malley *et al*. 1996). The results of the scatter plots of predicted vs. measured concentrations in the validation stage for the 61 soil samples indicated that the best prediction accuracies were obtained for the siderophile elements Ni, Cr and Co and the poorest prediction was for Cd by using VISNIR wavelength between 380 and 2500 nm. The correlation coefficients of determination R^2 value of Ni, Cr, Co and Cd are 0.81, 0.85, 0.80 and 0.20 respectively (Wu *et al*. 2008).

This result indicated that NIR technology can be used to predict cadmium concentrations using dry spmles of the tomato plants.

The eighth experiment showed in (Chapter 8). The standard sample was analysed using X-ray fluorescence spectrometer (Niton XL 3t 950 GOLDD+) in “mining mode” at the settings of (30 s main, 30 s low, 30 s high and 30 s light).

Thirty one ground stem samples representing genotype Grosse Lisse and Big Beef were analysed for Cd using wet digestion method and via XRF. The Cd concentrations from these methods correlated well (R^2=0.98) indicating that XRF is reliable and this method can be widely used for detecting Cd in plant tissues.

The XRF Cd readings correlated well with those derived from AAS, it was presumed that the XRF would provide similar prediction for other elements. Accordingly, the Cd concentrations of the 31 tomato tissues were correlated with the concentrations of other elements.

The results show that the accumulation of Cd in the shoots will also enhance the accumulation of Cl, S, K, Rb, Si and Ca. The Cd accumulation slightly enhanced the uptake of Mo and Zr, but had hardly any effect on the accumulation of Pd and Nb.

Chapter 10

Summary and Conclusions

10.1 Optimising tissue culture peocedures

This Chapter consisted of 3 experiments which addressed optimising procedures for growing plants in tissue culture and determining which of the plant tissues respond well to shoot induction.

The study revealed that very high level of germination can be obtained in tissue culture as most genotypes produced >90% germination. The results also showed that hypocotyls were better explants to use in tissue culture for shoot regeneration, in comparison with the cotyledons or shoot tips. Callus formation was found to be best when hypocotyls were used as explants.

Test with differing number of seeds (3, 6, 9 and 12) revealed that the use of 12 seeds per tube produced the highest biomass, but the seedlings were not as healthy as those found in treatments containing 3 seeds per tube. The test also showed the presence of large genotypic variation in shoot weight at various rates of seedling.

Comparison of different growth media revealed that agar medium was the best for producing plants with high biomass for the majority of the genotypes used. Thus, agar medium was used in subsequent experiments as opposed to sand or potting media.

10.2 Screening 25 genotypes for Cd tolerance and uptake

In this experiment 25 genotypes of tomato were tested for cadmium tolerance and accumulation in their shoots in tissue culture. Seven concentrations of Cd were used and 25 genotypes of tomatoes were grown for 5 weeks. Most of the genotypes were not affected by Cd (up to 500 µM) as far as their germination was concerned. However at 1000 µM, germination was affected. The plant height increased in many genotypes at low Cd (10, 30, 100 µM) but at higher Cd concentrations (1000 µM) seedling height declined in all genotypes. Toxicity symptoms were seen at high Cd levels. The highest cadmium accumulation was observed in Nash2 and Grosse Lisse treated with 1000 µM Cd. The highest Cd uptake was observed in CLt91t6D4, Grosse lisse and Nash1. Ten out of 25 genotypes could be used in phytoremediation of Cd contaminated sites and two of 25 genotypes showed the greatest potential to accumulate Cd in their shoots.

The results showed 18 out of 25 tomato genotypes were tolerant to high Cd (1000 µM). All 25 tomato genotypes were tolerant to low Cd levels (10, 30, 100, 200 µM Cd) as shown by similar shoot weight as in the control. Fifteen of the 25 tomato genotypes were hypersensitive to high Cd concentrations (1000 µM) and they were unable to show vertical growth and they died after 3 weeks of exposing them to Cd. Ten genotypes were tolerant and they also accumulated high Cd in their shoots.

10.3 Comparative tolerance of 4 genotypes of tomatoes in sand culture

Four genotypes of tomato were exposed to five concentrations of Cd (0, 30, 200, 500 and 1000 µM Cd) in sand culture in a glasshouse. Several seedlings were established at the start and 2-3 seedlings each were harvested from each pot in weeks 3, 5, 7 and

9 after adding Cd solution. The shoot height of Burke's Backyard and Grosse Lisse were not affected by 1000 µM Cd. Leaf and stem dry weights of all genotypes were compared for each of the harvesting date. It was found that only the 1000 µM Cd treatment had reduced shoot biomass (Fig 6.5), indicating resilience of these genotypes to tolerant Cd. The proportion of leaf and stem did not change with the Cd treatment for most genotypes at most harvest times (exceptions were week 3 which had higher proportions of leaf than stem). The highest Cd concentration was found at 1000 µM Cd and the uptake rate of leaves was found to be much higher than that of stems. The genotypes Big Beef and Burke's Backyard accumulated the most Cd in their shoots so they could be used in phytoremediation of cadmium polluted soils.

10.4 Long term response of two genotypes of tomato to Cd in a glasshouse

A glasshouse experiment was conducted using two genotypes of tomato to test for distribution of Cd in their shoots. Tomato plants were grown in sand culture in a glasshouse and were exposed to three Cd concentrations for up to 17 weeks. Cadmium (0, 100 and 500 µM) was added after seed germination. The responses of the genotypes Grosse Lisse and Big Beef to Cd varied with the Cd levels used. The leaf number and shoot height of Grosse Lisse was not affected by 500 µM Cd. Shoot dry weight of Big Beef was reduced at 500 µM Cd after 7 weeks but not at 17 weeks Exposure of plants to Cd did not affect chlorophyll content in both genotypes. The results showed that the highest leaf Cd concentration was found in Big Beef after 7 weeks (366µg/g). The stem Cd concentration was not different amongst the two genotypes at 500 µM after 7 weeks, and fewer differences were noted after 17 weeks. The leaves of Grosse Lisse had 2 times higher Cd uptake than Big Beef at 500 µM Cd at 7 weeks and 17 weeks. The stem Cd uptake was 6 times higher in both

genotypes after 17 weeks than at 7 weeks in 500 µM Cd. This suggests that the stems may continue to accumulate Cd over time, and longer exposure of plants may lead to greater removal of Cd from the soil.

10.5 Use of NIR and XRF to detect cadmium concentrations in tomato tissues

Near infrared spectroscopy based on effective wavelengths and chemometrics was used to determine cadmium concentration in the stems of four genotypes. Cadmium reference values were obtained by atomic absorption spectrometry and these values were regressed against NIR spectral transformations using partial least squares regression methods. The NIR predicted cadmium concentration was highly correlated with the AAS measured cadmium concentration (R^2=0.99). These results suggested that the NIR spectrometry technique could provide an accurate, reliable and non destructive method of predicting cadmium concentrations of tomato stems.

X-ray fluorescence analysis was also applied to determine cadmium concentrations of ground tomato stems. Stems of two genotypes viz., Grosse Lisse and Big Beef were used and the plants were harvested at 7 weeks and 17 weeks after exposing them to 100 and 500 µM Cd. The ground stem tissues were analysed by XRF spectroscopy. The results showed very high correlation between the results of XRF determined Cd concentration and AAS measured Cd concentrations. The results of both NIR and XRF spectroscopy tests clearly demonstrate that either of the techniques can be used to detect Cd concentrations of dried grounded plant tissues. Since these techniques can determine metal concentrations from intact samples, they will have an added advantage, as this process could reduce the costs involved in digesting plant samples using acids and then running these samples through AAS or

ICP. While the accuracy of the NIR and XRF may be questioned, as compared to the other techniques, but for rapid detection of chemicals in plants and soils, the NIR and XRF techniques seem to provide reliable information.

10.6 Future Research

Tomato is one of the most popular vegetable crops in the world and is usually grown around the cities where the soils are likely to be contaminated with heavy metals like cadmium. To minise the chances of heavy metals entering into the food chain, it is important that the semiurban soils are kept free from heavy metals. In this connection, phytoremediation can offer a useful solution, as it is economical and easy to apply in the field. However, the success of this technique relies upon the selection of appropriate species and genotypes that can accumulate heavy metals in their shoots.

The current research has screened 25 genotypes of tomato (commercial cultivars and wild genotypes), which culminated in identifying genotypes that grow well in cadmium contaminated soils and accumulate high levels of cadmium in their tissues. Some of these cultivars can be readily used in the cleanup of Cd contaminated soils. Some wild genotypes that accumulate high concentrations of Cd and sparingly produce fruits may be better suited for phytoremediation than commercial cultivars, as the risks of fruit containing Cd can be minimised. Alternatively, such genotypes can be used to hybridise with other genotypes, with the view to producing high shoot biomass, no fruits and high Cd uptake rates into their shoots.

Study such as the identification of plants that have deeper root system, stress tolerance and those producing very few flowers to be initiated. Furthermore,

potential use of these species in the cleanup of a known contaminated soil in the field must be demonstrated with the view to promoting phytoremediation techniques.

Study on translocation patterns of Cd and other heavy metals into fruits and seeds of commercial species (edible) would be desirable to assess the impact of heavy metals on food chain.

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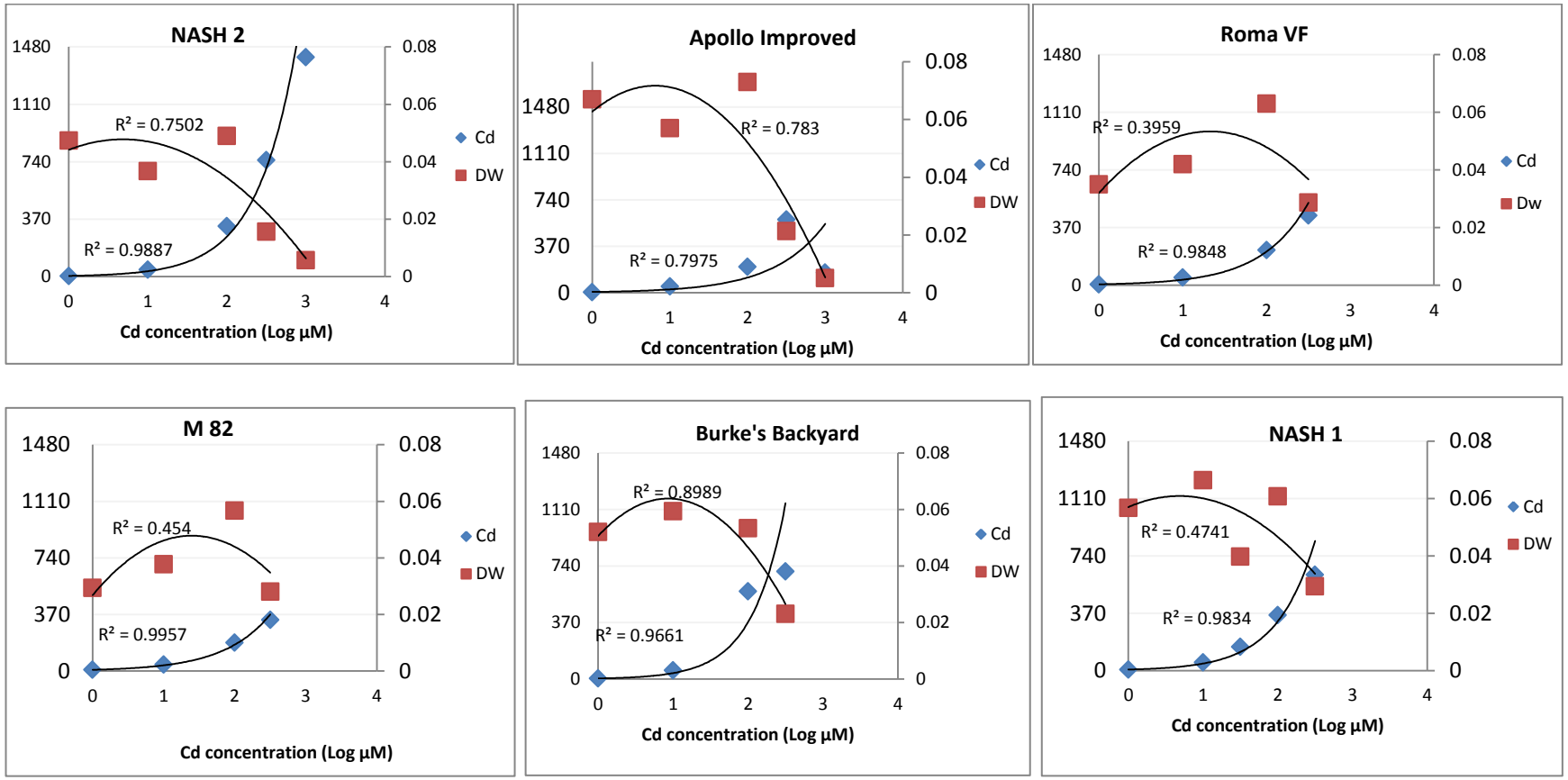
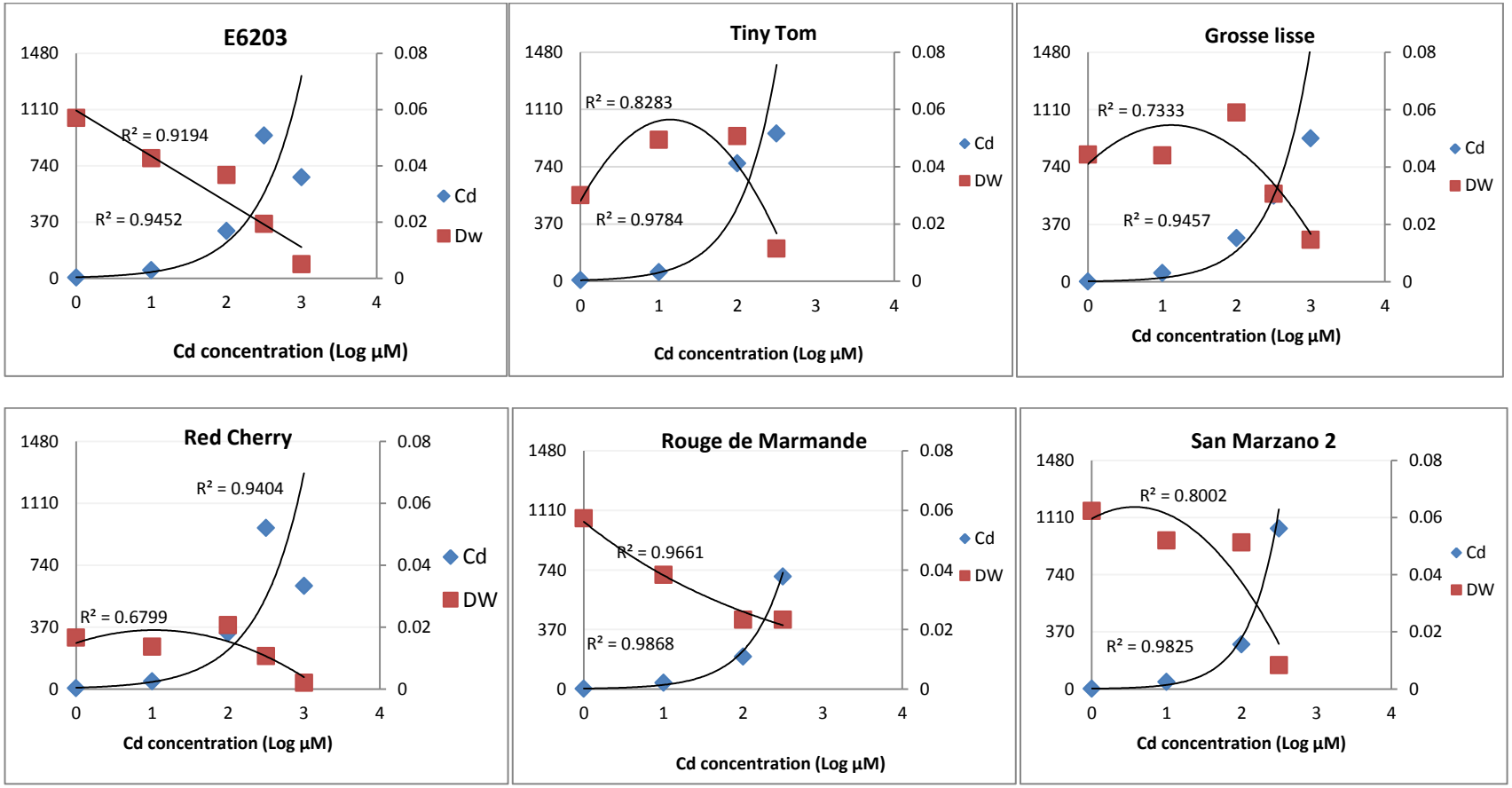
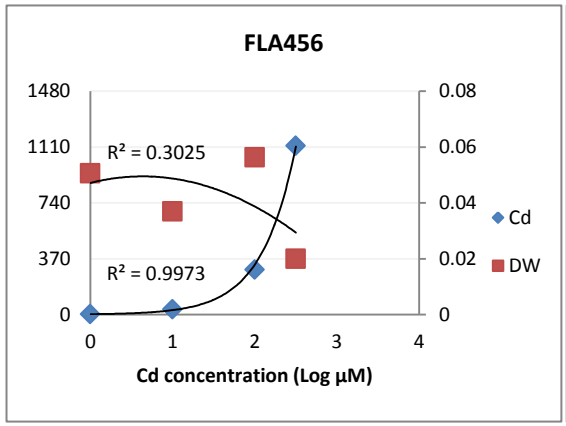
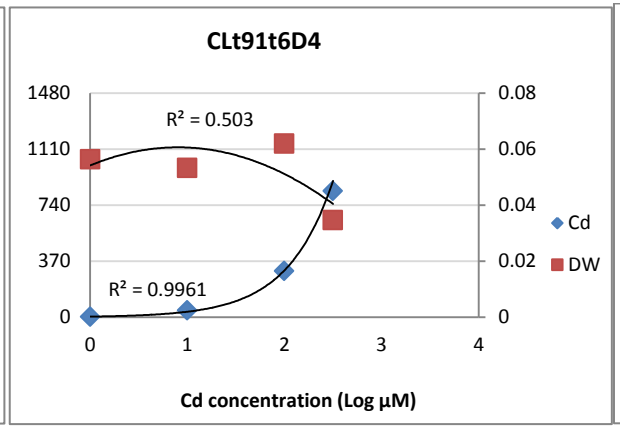
APPENDICES

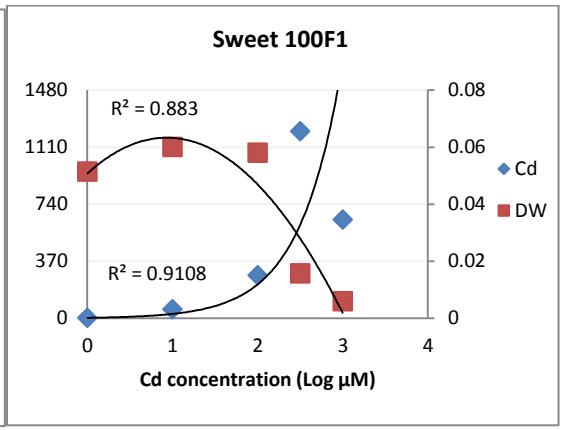
Appendix1 Leaf cadmium concentrations and leaf dry weight in 24 genotypes of tomato grown in tissue culture at 0, 10, 30, 100, 200, 500 and 1000 µM cadmium.

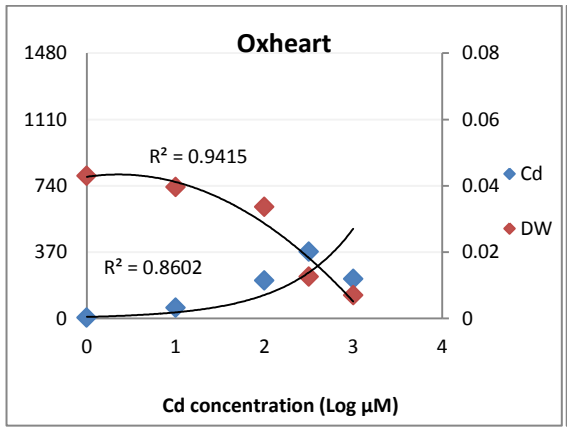


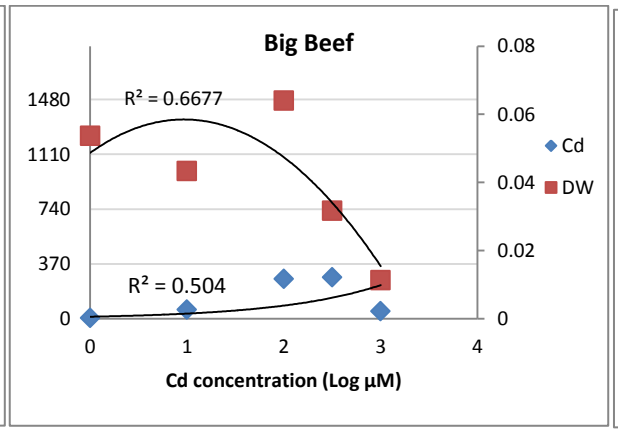

E6203
1480
1110
740
370
0
0.08
0.06
0.04
0.02
0
R² = 0.9194
R² = 0.9452
Cd
Dw
0
1
2
3
4
Cd concentration (Log µM)
Tiny Tom
R² = 0.8283
R² = 0.9784
Cd
DW
Cd concentration (Log µM)
Grosse lisse
R² = 0.7333
R² = 0.9457
Cd
DW
Cd concentration (Log µM)
Red Cherry
R² = 0.9404
R² = 0.6799
Cd
DW
Cd concentration (Log µM)
Rouge de Marmande
R² = 0.9661
R² = 0.9868
Cd
DW
Cd concentration (Log µM)
San Marzano 2
R² = 0.8002
R² = 0.9825
Cd
DW
Cd concentration (Log µM)

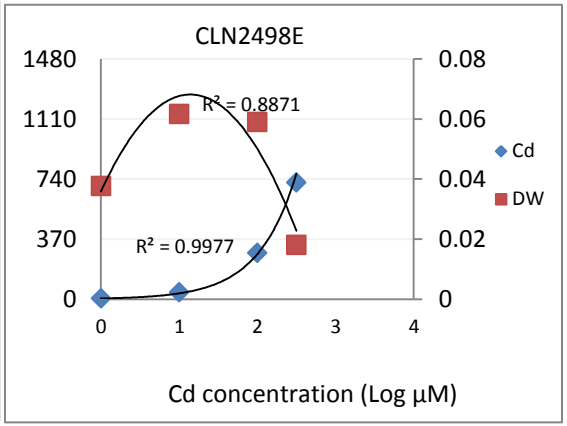

FLA456
1480
1110
740
370
0
0.08
0.06
0.04
0.02
0
R² = 0.3025
R² = 0.9973
Cd
DW
0
1
2
3
4
Cd concentration (Log µM)

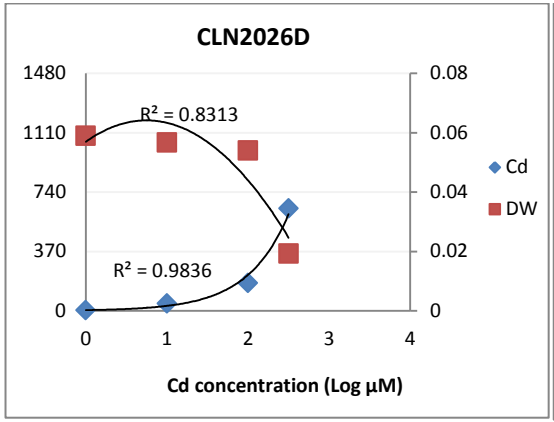

CLt91t6D4
1480
1110
740
370
0
0.08
0.06
0.04
0.02
0
R² = 0.503
R² = 0.9961
Cd
DW
0
1
2
3
4
Cd concentration (Log µM)

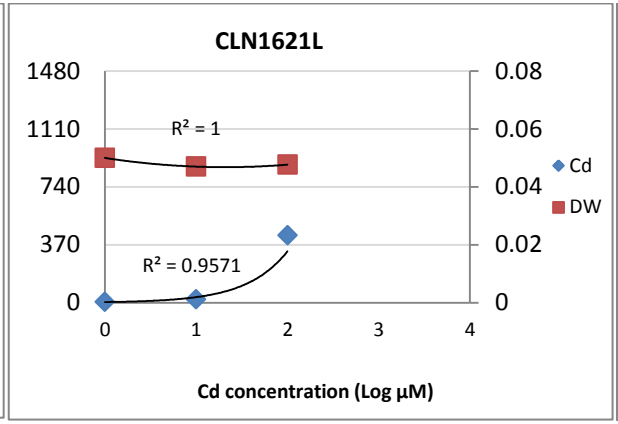

Sweet 100F1
1480
1110
740
370
0
0.08
0.06
0.04
0.02
0
R² = 0.883
R² = 0.9108
Cd
DW
0
1
2
3
4
Cd concentration (Log µM)

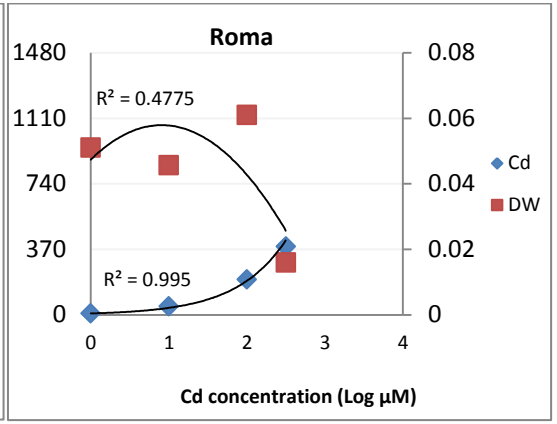

Oxheart
1480
1110
740
370
0
0.08
0.06
0.04
0.02
0
R² = 0.9415
R² = 0.8602
Cd
DW
0
1
2
3
4
Cd concentration (Log µM)

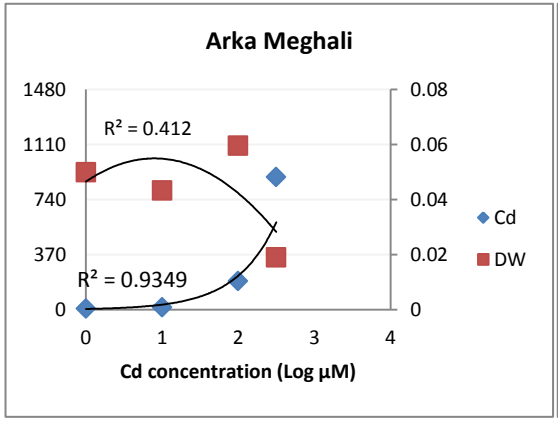

Big Beef
1480
1110
740
370
0
0.08
0.06
0.04
0.02
0
R² = 0.6677
R² = 0.504
Cd
DW
0
1
2
3
4
Cd concentration (Log µM)

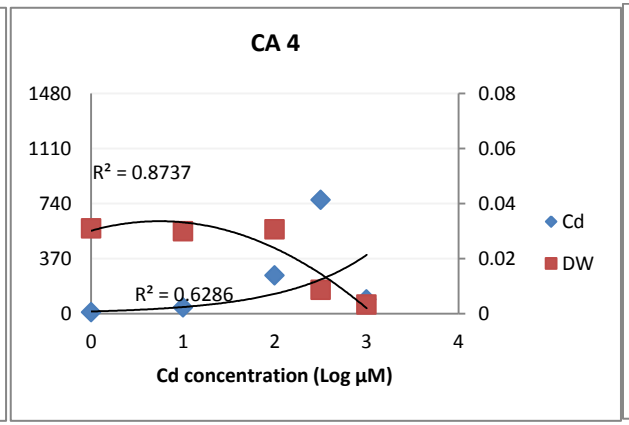

CLN2498E
1480
1110
740
370
0
0.08
0.06
0.04
0.02
0
R² = 0.8871
R² = 0.9977
Cd
DW
0
1
2
3
4
Cd concentration (Log µM)

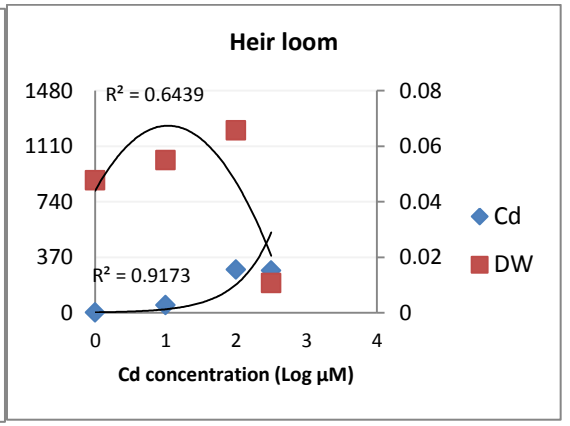

CLN2026D
1480
1110
740
370
0
0.08
0.06
0.04
0.02
0
R² = 0.8313
R² = 0.9836
0 1 2 3 4
Cd concentration (Log µM)
Cd
DW


CLN1621L
1480
1110
740
370
0
0.08
0.06
0.04
0.02
0
R² = 1
R² = 0.9571
0 1 2 3 4
Cd concentration (Log µM)
Cd
DW

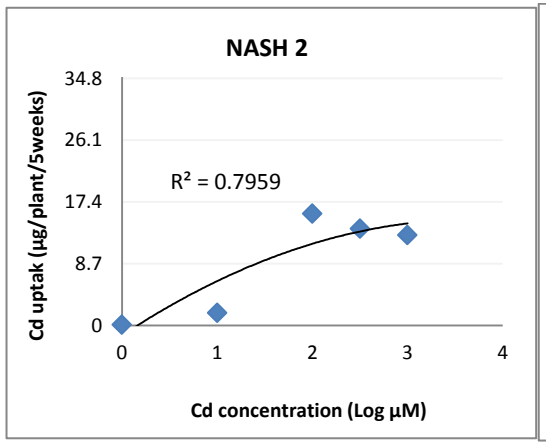

Roma
1480
1110
740
370
0
0.08
0.06
0.04
0.02
0
R² = 0.4775
R² = 0.995
0 1 2 3 4
Cd concentration (Log µM)
Cd
DW

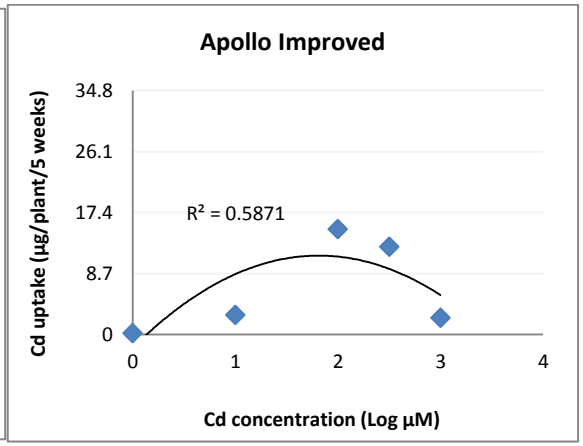

Arka Meghali
1480
1110
740
370
0
0.08
0.06
0.04
0.02
0
R² = 0.412
R² = 0.9349
0 1 2 3 4
Cd concentration (Log µM)
Cd
DW

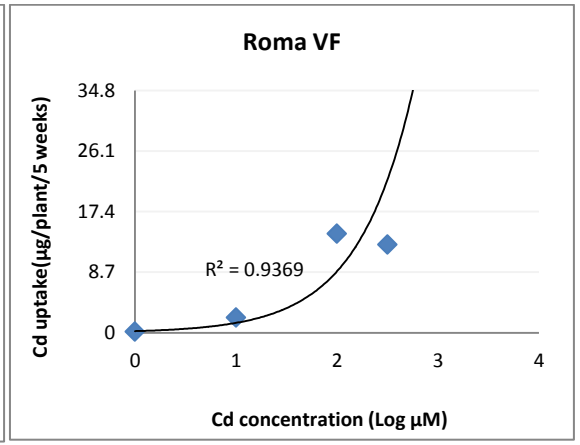

CA 4
1480
1110
740
370
0
0.08
0.06
0.04
0.02
0
R² = 0.8737
R² = 0.6286
0 1 2 3 4
Cd concentration (Log µM)
Cd
DW

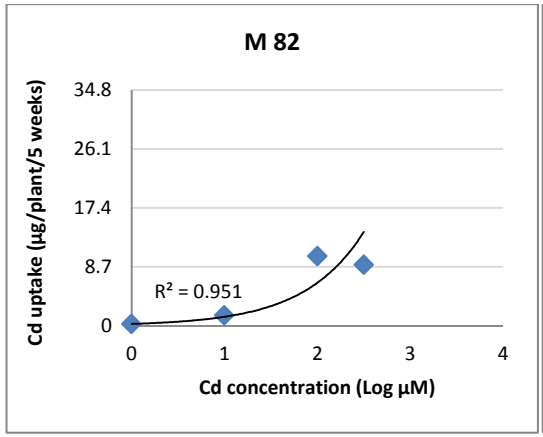

Heir loom
1480
1110
740
370
0
0.08
0.06
0.04
0.02
0
R² = 0.6439
R² = 0.9173
0 1 2 3 4
Cd concentration (Log µM)
Cd
DW

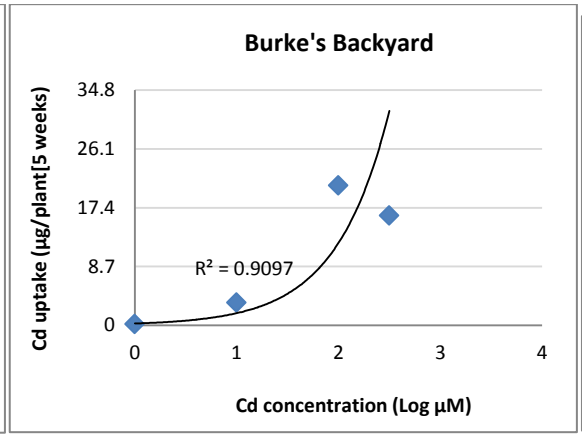
Appendix 2 Cadmium uptake (µg/g dw/5 wks) in 24 genotypes of tomato grown in tissue culture at 0, 10, 30, 100, 200, 500 and 1000 µM cadmium.

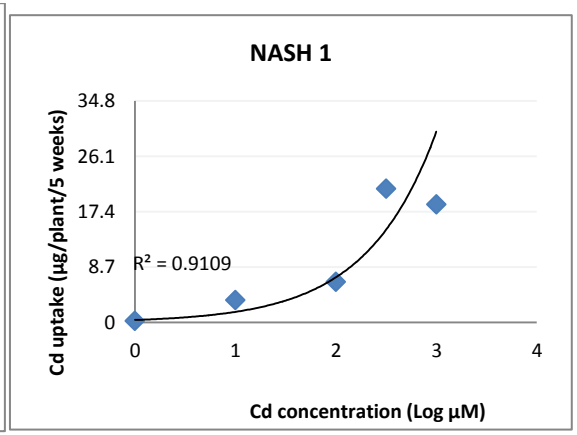


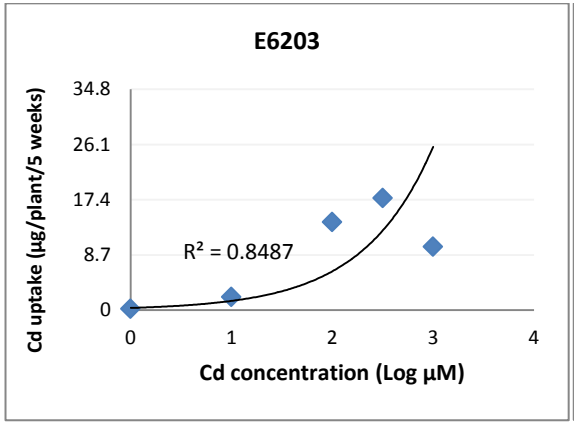


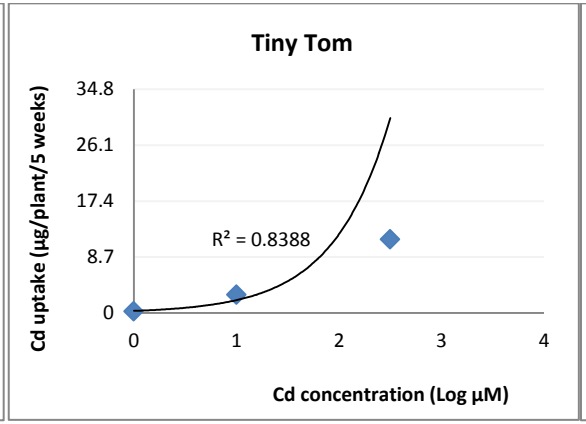


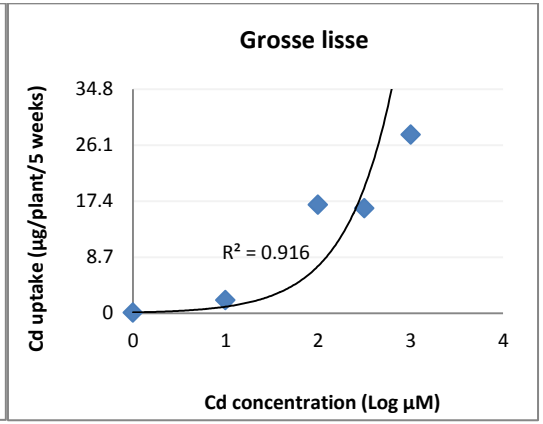


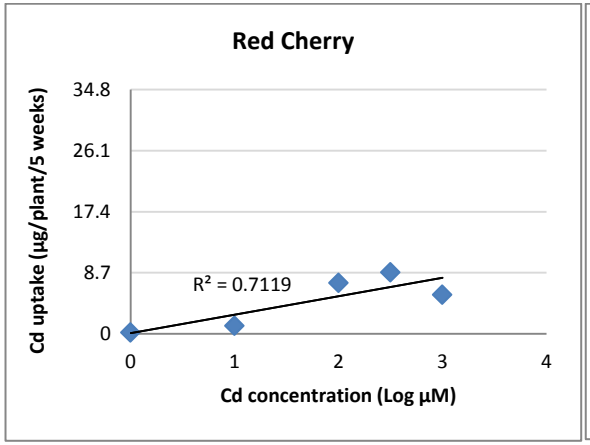


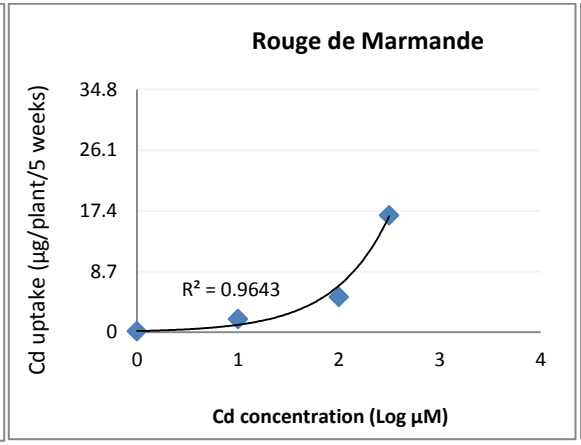


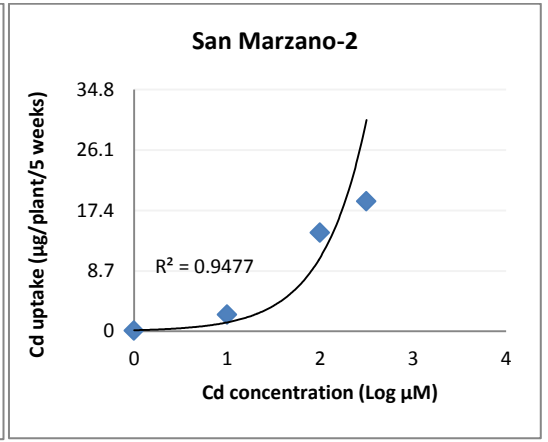

E6203
Cd uptake (µg/plant/5 weeks)
34.8
26.1
17.4
8.7
0
R² = 0.8487
0
1
2
3
4
Cd concentration (Log µM)

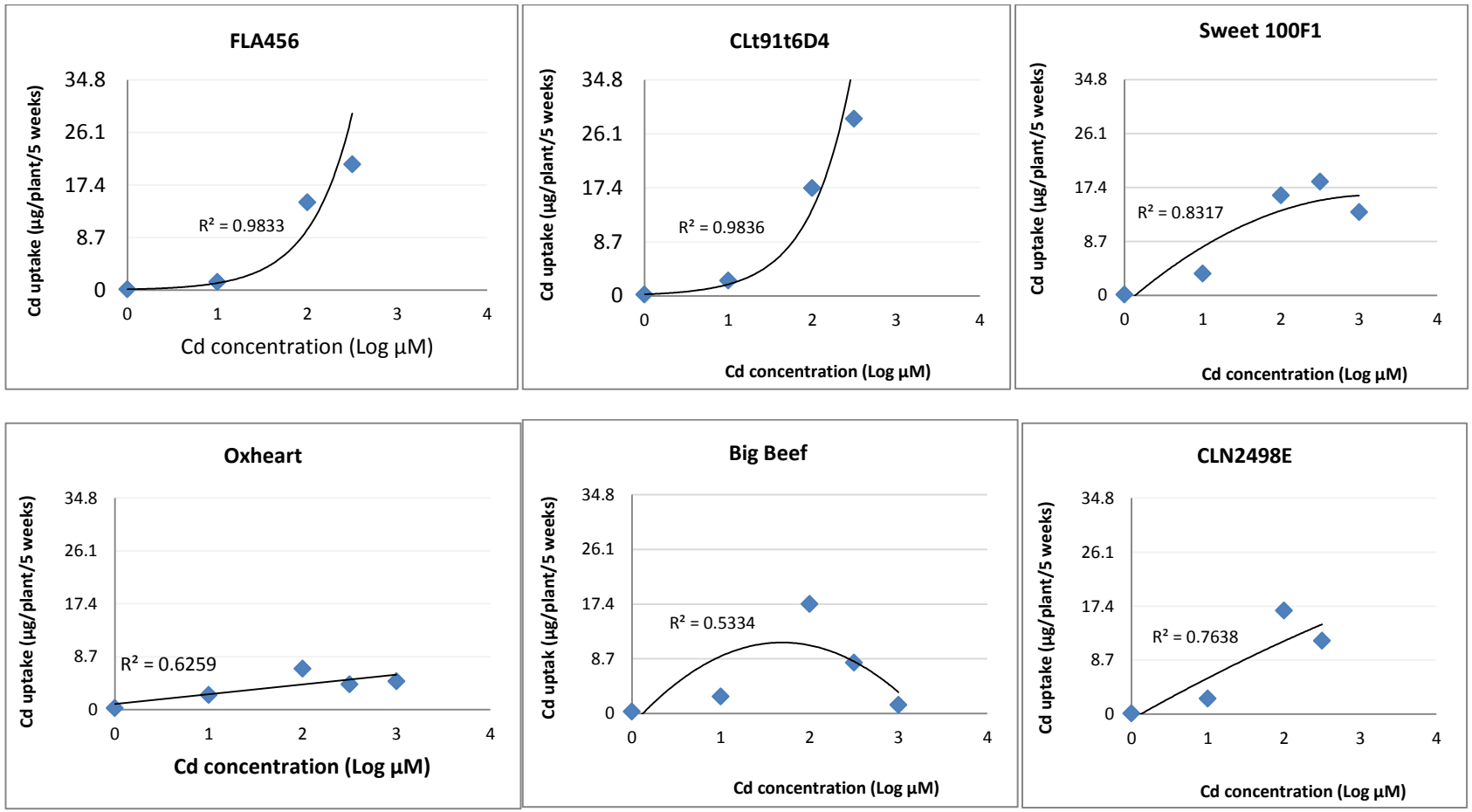

Tiny Tom
Cd uptake (µg/plant/5 weeks)
34.8
26.1
17.4
8.7
0
R² = 0.8388
0
1
2
3
4
Cd concentration (Log µM)

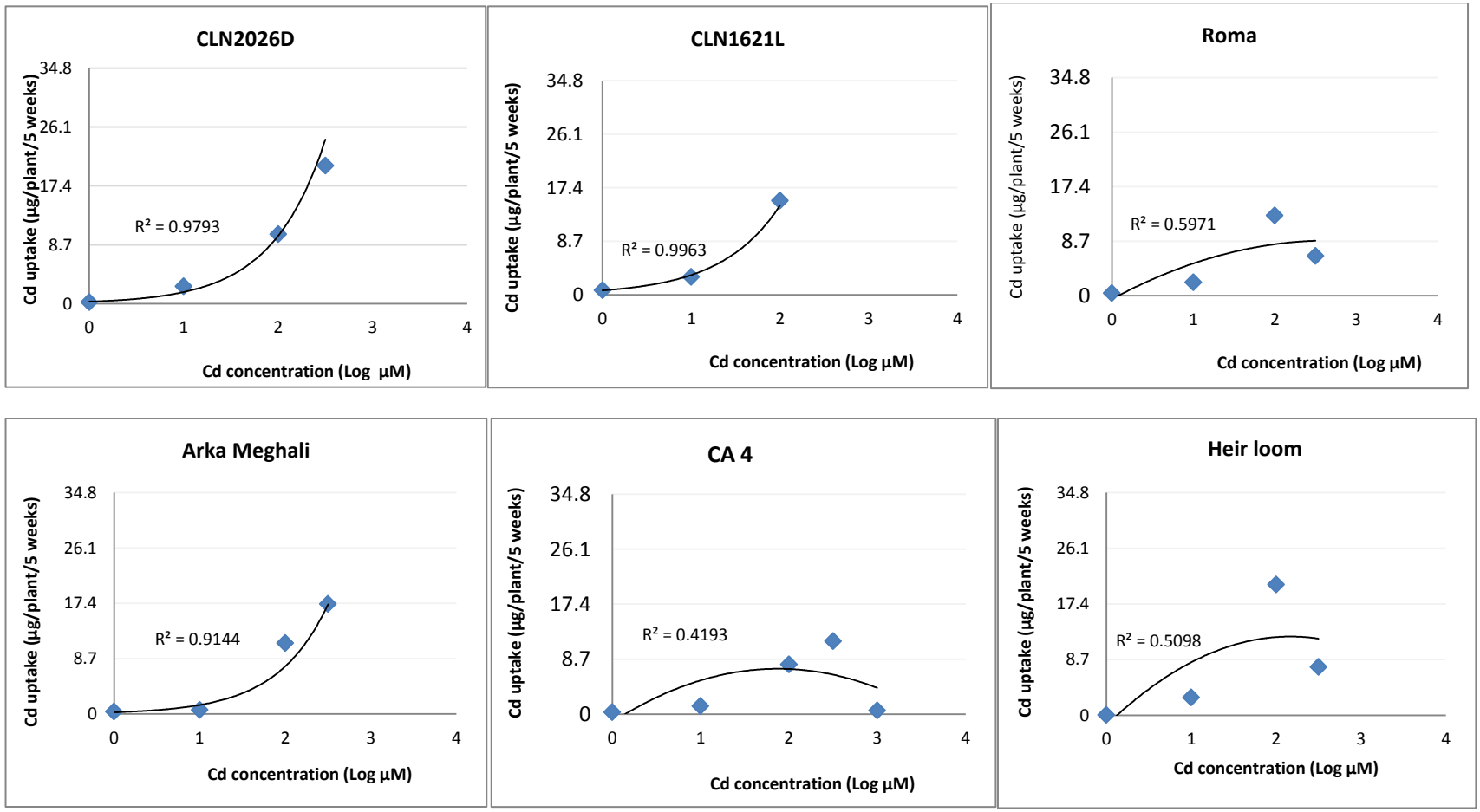

Grosse lisse
Cd uptake (µg/plant/5 weeks)
34.8
26.1
17.4
8.7
0
R² = 0.916
0
1
2
3
4
Cd concentration (Log µM)


Red Cherry
Cd uptake (µg/plant/5 weeks)
34.8
26.1
17.4
8.7
0
R² = 0.7119
0
1
2
3
4
Cd concentration (Log µM)


Rouge de Marmande
Cd uptake (µg/plant/5 weeks)
34.8
26.1
17.4
8.7
0
R² = 0.9643
0
1
2
3
4
Cd concentration (Log µM)


San Marzano-2
Cd uptake (µg/plant/5 weeks)
34.8
26.1
17.4
8.7
0
R² = 0.9477
0
1
2
3
4
Cd concentration (Log µM)


FLA456
Cd uptake (µg/plant/5 weeks)
34.8
26.1
17.4
8.7
0
R² = 0.9833
0
1
2
3
4
Cd concentration (Log µM)
CLt91t6D4
Cd uptake (µg/plant/5 weeks)
34.8
26.1
17.4
8.7
0
R² = 0.9836
0
1
2
3
4
Cd concentration (Log µM)
Sweet 100F1
Cd uptake (µg/plant/5 weeks)
34.8
26.1
17.4
8.7
0
R² = 0.8317
0
1
2
3
4
Cd concentration (Log µM)
Oxheart
Cd uptake (µg/plant/5 weeks)
34.8
26.1
17.4
8.7
0
R² = 0.6259
0
1
2
3
4
Cd concentration (Log µM)
Big Beef
Cd uptak (µg/plant/5 weeks)
34.8
26.1
17.4
8.7
0
R² = 0.5334
0
1
2
3
4
Cd concentration (Log µM)
CLN2498E
Cd uptake (µg/plant/5 weeks)
34.8
26.1
17.4
8.7
0
R² = 0.7638
0
1
2
3
4
Cd concentration (Log µM)


CLN2026D
Cd uptake (µg/plant/5 weeks)
34.8
26.1
17.4
8.7
0
R² = 0.9793
0 1 2 3 4
Cd concentration (Log µM)
CLN1621L
Cd uptake (µg/plant/5 weeks)
34.8
26.1
17.4
8.7
0
R² = 0.9963
0 1 2 3 4
Cd concentration (Log µM)
Roma
Cd uptake (µg/plant/5 weeks)
34.8
26.1
17.4
8.7
0
R² = 0.5971
0 1 2 3 4
Cd concentration (Log µM)
Arka Meghali
Cd uptake (µg/plant/5 weeks)
34.8
26.1
17.4
8.7
0
R² = 0.9144
0 1 2 3 4
Cd concentration (Log µM)
CA 4
Cd uptake (µg/plant/5 weeks)
34.8
26.1
17.4
8.7
0
R² = 0.4193
0 1 2 3 4
Cd concentration (Log µM)
Heir loom
Cd uptake (µg/plant/5 weeks)
34.8
26.1
17.4
8.7
0
R² = 0.5098
0 1 2 3 4
Cd concentration (Log µM)