2013 | Faculteit Wetenschappen

DOCTORAATSPROEFSCHRIFT

Effects of cadmium on the proteome of roots and leaves of poplar: comparison between hydroponically and soil grown plants

Proefschrift voorgelegd tot het behalen van de graad van doctor in de wetenschappen, biologie, te verdedigen door:

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D/2013/2451/18
ISBN 9789089130242

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KNOWLEDGE IN ACTION

One of the main questions is "how can one determine the FULL proteome of an organism". The answer is simple, although shocking for the least aware:
" you can NOT".
- Lemos *et al.*, 2010 -

Les pappillons ne comptent pas les mois, mais les instants

Een theorie is maar geldig tot het tegendeel bewezen is
- Karl Popper -

PhD thesis presented on the 31th of May 2013 at Hasselt University

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Woord vooraf

Een werk als dit is moeilijk alleen te voltooien. Tijdens mijn doctoraat heb ik vele mensen leren kennen die me hebben geholpen bij het volbrengen van deze taak. Graag begin ik met het bedanken van mijn promotoren:

Jaco, aan het prille begin van mijn carrière als bioloog heb jij me, samen met je collega-docenten, de basiskennis bijgebracht. Na mijn kandidaturen moest ik echter verhuizen naar een andere universiteit. Na heel wat wikken en wegen, werd het de Universiteit Antwerpen… maar mijn hart bleef verkocht aan het gezellige Diepenbeek. Hoewel er in Antwerpen een mogelijkheid was om te doctoreren, wou ik toch graag terugkeren naar mijn 'thuisbasis'. Aldus besloot ik u te contacteren en er werd al snel een afspraak gemaakt om de mogelijkheden te bekijken. Vanaf toen ging alles is een razend tempo vooruit… Ik mocht op sollicitatiegesprek komen voor een BOF project dat zich richtte op onderzoek naar fytoremediatie met proteomics als aangewende techniek. Als pas afgestudeerde maakte ik me klaar voor het gesprek… niet volledig beseffende dat dit een "echt" sollicitatiegesprek ging zijn met een hele jury tegenover mij, begon ik mijn trip naar Diepenbeek. Die dag zal altijd in mijn geheugen gegrift blijven omdat ik waarschijnlijk nooit meer zal kunnen herhalen wat er toen gebeurd is: naar een sollicitatie gesprek gaan met een gescheurde jeans en enkele dagen laten te horen krijgen dat ik het team mocht komen versterken ☺! Bedankt voor het vertrouwen dat je in mij stelde, want op basis van de looks moest je die dag echt niet afgaan! Maar ook tijdens mijn doctoraat kon ik steeds bij je terecht en heb ik steeds alle kansen gekregen om me volledig te ontplooien als onderzoeker. Een oprechte dank je wel hiervoor!

Ann, als ik aan jou terugdenk, moet ik altijd lachen… De manier waarop jij iemand gek kan maken voor je vak, je theorieën of ideeën zijn onwaarschijnlijk! Ik herinner me nog perfect hoe jij op het bord genetica oefeningen stond uit te leggen en ik dacht "nauw… dit is best nog leuk"! Enkele maanden geleden nog kwam ik bij jou op de bureau met enkele ideeën die ik had

ontwikkeld tijdens het schrijven van mijn meta-analyse. Hoewel ik wel achter de ideeën stond, was ik toch een beetje onzeker over hoe ze onthaald zouden worden. Maar na ons gesprek was ik zo euforisch dat ik me dadelijk achter mijn computer heb gezet en de hele conclusie in één adem heb neergeschreven! Bedankt om mij te laten genieten van deze geweldige eigenschap die jij hebt en raak ze alsjeblieft nooit kwijt!

Tijdens mijn doctoraat heb ik de kans gekregen met een hoop toffe mensen samen te werken.

Nele, bedankt om mij de eerste dag op te vangen en met mij alle bureaus af te gaan om de mensen te leren kennen. Ook bedankt om me in te wijden in de wereld van de endofyten en me kennis te laten maken met de diverse protocols die hierbij kwamen kijken. Ook tijdens de periode dat de stekken moeilijk deden, heb je mee blijven zoeken naar een oplossing. Bedankt voor alle steun en uiteraard wens ik je veel succes in de toekomst!

Karen V wil ik speciaal bedanken om me te introduceren in de proteomics wereld. Al snel stond ik met haar in het labo om voor de eerste keer de hele 2DE workflow te doorlopen. Het was een zeer leerrijke ervaring! Vanaf het begin heb je me gewaarschuwd voor de techniek met de woorden 'bezint eer ge begint'. Met veel bewondering heb ik je super hard zien werken tijdens de laatste fase van je doctoraat, maar zelfs toen besefte ik nog niet volledig waar je het over had. Pas toen ik de literatuur begon te raadplegen om een antwoord te kunnen formuleren op de vaak gestelde vraag "wat verwacht je dat zal gebeuren", kwam ik tot inzicht. Hoofdstuk 4.2 is dan ook speciaal aan jou opgedragen! Want jij was, misschien zonder dat je het besefte, een enorme inspiratiebron voor dit schrijven! Bedankt voor alles en hopelijk komen we elkaar nog eens tegen!

Sascha, jou wil ik bedanken voor het luisterend oor dat je bood. Onze bureau kwam vaak van pas om even alle frustratie van het labo achter ons te kunnen laten en om alles terug te kunnen relativeren! Gezien mijn perfectionisme en soms iets te gedreven aard, heb ik daar wel vaak

nood aan gehad ☺ Een oprechte dank je wel is dan ook niet misplaatst! Ik wens je nog heel veel succes met je doctoraat en met alles wat er je nog te wachten staat.

Sacha, the first time we met wasn't a big succes... At ProteomLux I presented my first poster revealing the results of my optimization protocol. What I didn't know was that I included your extraction protocol in the optimization analysis. Since it didn't rank the best, I soon had a lot of (negative?) attention of the organizing committee ☺ On top, you all thought that that "Joke-guy" was a man... well, I suppose you were disappointed when meeting me! On the other hand... we managed to get along despite our differences and up to date, no harm was done. Serious now, I want to thank you for coming to the lovely Belgian country to teach me the critical points of working with CyDyes and analysing the results! But I think a sincere "thank you" from your side is not inappropriate as well... If you didn't came to Belgium to teach me the art of CyDyes, you might not have met your wonderful wife! ☺ Hope your Indian wedding will be beautiful and I wish you all the best in your further life!

Jan W en Michiel wil ik graag bedanken voor wie ze zijn. Om altijd bij hun terecht te kunnen, om niet rond de pot te draaien, om te durven vragen of alles wel OK is met me en om te zien wie ik echt ben! Ik heb hele goede gesprekken met jullie gehad en dat heeft me door de zware periodes geholpen. Jan, ik wens je nog super veel succes met het schrijven en laat me weten wanneer je verdediging is, zodat ik kan komen supporteren! Doe Batu nog een dikke kus van me! Michiel, je had het bijna verknald toen je zei dat ik geen "meisje-meisje" was, maar zie hier... een speciale vermelding in mijn dankwoord!!

Michelle, ooooh Michelle, er staat nog een SNOOPY-mok op jou te wachten ☺ Meid, bedankt voor de vele babbels en voor je luisterend oor! Ik heb een super tijd met jou gehad en ik geloof in je! Ik hoop dat we elkaar nog vaak mogen zien! Veel succes met je doctoraat en weet dat je altijd bij mij terecht kan!!

Natuurlijk wil ik alle leden van de bacteriëngroep speciaal bedanken voor alle medewerking, de vele vergaderingen waarin we ideeën konden uitwisselen en om een super leuk team te vormen waarin ik heb kunnen werken.

Ann W en Carine… Goh, als ik begin op te noemen waarvoor ik jullie moet bedanken, denk ik dat ik nog wel een tijdje bezig kan zijn! Ik was altijd welkom bij jullie voor vragen, voor een babbel, voor een helpende hand bij de oogst,… Kortom, ik heb nooit een "nee" van jullie gehoord! Ook al is het misschien jullie job, naar mijn mening doen jullie meer dan dat! Vandaar ook de merci'tjes op tijd en stond ☺ Bedankt, om er altijd te zijn!

Jan D wil ik ook bedanken voor alle hulp in de serre en daarbuiten! En natuurlijk voor de paardenpraatjes en om mee te komen genieten van mijn jumpings ☺ Paardenmensen weten elkaar toch altijd te vinden he! Jan, ik wens je heel goede gezondheid toe en hoop dat je snel terug met de paardjes bezig kan zijn!

Robert, bedankt voor het coördineren van de vele metaalbepalingen tijdens mijn doctoraat. Tom en Frank, ik wil jullie allebei bedanken voor de steun en het vertrouwen dat ik in jullie heb mogen stellen! Dennis, bedankt voor de leerrijke voormiddag ;) Hopelijk ga ik er nog gebruik van kunnen maken!

Verder wil ik ook Prof. Jean-Paul Noben bedanken voor het analyseren en valideren van de eiwit identificaties. Een speciale dank je wel ook aan Erik Royackers om mij de kneepjes van eiwit digestie te leren en zoveel tijd in de identificaties te steken!

Toen ik in 2008 begon aan mijn doctoraat, waren we met een "kleine" groep. Tijdens de 5 jaar dat ik in jullie team heb kunnen werken, zijn we in snel tempo gegroeid! Het is dus niet mogelijk om iedereen die ik tijdens mijn doctoraat ontmoet heb als collega te kunnen bedanken. Maar ik wil dat jullie allemaal weten dat jullie me allemaal geholpen hebben! Was het nu de lach in de gang, het openhouden van een deur, het helpen verslepen van zakken zand, … het

heeft me allemaal wat gedaan en al die kleine beetjes maakten de moeilijke periodes draaglijk! Het was leuk om in zo een aangename sfeer te kunnen werken!
Natuurlijk wil ik ook het IWT-Vlaanderen (Agentschap voor Innovatie door Wetenschap en Technologie) bedanken om mij een doctoraatsbeurs (81069) te schenken. Zonder hun financiele steun was dit project onmogelijk!
De leden van de jury wil ik bedanken voor hun kritische evaluatie van dit proefschrift. I would like to thank all members of the jury for their time and contribution to this PhD: 'Effects of cadmium on the proteome of roots and leaves of poplar: Comparison between hydroponically and soil grown plants'.
Natuurlijk wil ik ook mijn ouders bedanken. Mama en papa, bedankt om me altijd te steunen! Wat ik ook doe, waar ik ook ben, ik zal altijd op jullie kunnen rekenen. Ik wil dat jullie weten dat ik dit niet als vanzelfsprekend beschouw! Laura, Evelyn, Sofie, Eline en Gudrun, ooit heeft iemand gezegd 'als je een hand vol échte vrienden hebt, mag je jezelf zeer gelukkig prijzen'. Wel, jullie zijn mijn 'handje vol'! Bedankt voor alles! Tenslotte wil ik ook mijn vriend bedanken om er altijd voor me te zijn, om me steeds weer proberen aan het lachen te krijgen, om me mezelf te laten zijn en me soms te dwingen het eventjes allemaal los te laten.
Ik weet nu al, het komt wel goed.! Si ~ Fly ~Prego ~ De Nada ^^

Joke Dupae

Mei 2013

agentschap voor Innovatie
door Wetenschap en Technologie

Summary

Atmospheric deposits originating from zinc smelters resulted in large scale diffuse metal contamination in North Eastern Belgium. Since toxic metals, like cadmium (Cd), can pose severe risks to human health, threshold values have been defined for soils and crops. Exceeding these threshold values and consequently loosing these economic valuable soils, enhances the authorities' interest in alternative remediation strategies. Classical remediation techniques (e.g. soil washing, excavation and dumping) are expensive, destructive for soil structure and microorganisms and not applicable for large scale and diffuse contaminations. Phytoextraction, the *in situ* use of plants to extract contaminants from contaminated soils, is believed to be a promising alternative for metal contaminated sites. However, to be economically attractive the phytoextraction process needs to be improved. In case of organic contaminants, equipping plant endophytes with appropriate degradation pathways, resulted in a decrease of phytotoxicity and evapotranspiration and, by consequence, an enhanced remediation efficiency. This raised the question if equipping plant endophytes with metal sequestration systems might improve efficiency of metal phytoextraction as well.

In section 3, two plant growth-promoting bacteria were equipped with the CZR operon, which enables bacteria to bind Cd onto their cell wall. Based on phenotypical traits, transconjugant strain W1366-5 seemed most promising to enhance phytoextraction of Cd contaminated soils. However, the results from the greenhouse experiment suggested that transconjugant strain E1600-11 is more promising *in planta* under Cd-exposed conditions (chapter 3.1).

Since obvious differences in plants' toxicity responses to Cd were observed in hydroponical and soil cultivation systems, a more in depth examination of the underlying mechanisms of Cd toxicity was conducted using a proteomic approach. Therefore, first an optimization of the proteomics workflow was performed (chapter 4.1). Subsequently, a literature study was conducted to get more insights into the already described effects of Cd on the plants' proteome; from this study, it became obvious that a comparison between proteomics experiments is not that straightforward. Therefore, encountered obstacles were discussed in a meta-analysis (chapter 4.2). Further, a comparison was made between hydroponic and soil cultivation systems indicating that differences in Cd responses between both cultivation systems might exist. However, since a huge diversity in experimental set

ups and used materials was present, this hypothesis cannot be confirmed or rejected based on the meta-analysis data.

In order to address the question whether an extrapolation from hydroponically obtained data towards soil grown plants is possible, an experiment was conducted in which poplar cuttings were grown on both, hydroponic (chapter 4.3) and soil (chapter 4.4) cultivation systems. Except for the cultivation system, other parameters were kept identical in these experiments. Their response to Cd was examined by comparing control cuttings to Cd-exposed cuttings after 3 and 21 days of exposure.

Comparing the proteomic data from our hydroponics experiment (chapter 4.3) to those extracted from hydroponic cultivation experiments in the meta-analysis, revealed a general down-regulation of CO_2-fixation, chloroplast electron transport and ATP synthases in leaves. However, contrasting responses were present for the stress metabolism. In roots, the same metabolic pathways were affected in our experiment compared to those present in the meta-analysis, though a closer look revealed some differences.

The general down-regulation of CO_2-fixation, chloroplast electron transport and ATP synthases present in soil grown leaves when studying the meta-analysis data, was not confirmed in our soil grown experiment (chapter 4.4). As for the hydroponic cultivation experiment, the same metabolic pathways were addressed in soil grown roots of our experiment compared to those described in the meta-analysis. However, a closer look again revealed some dissimilarities. A comparison between both experiments (chapter 4.5) revealed dissimilarities in the plants' response to Cd. Moreover, it was observed that an effect time was minor in hydroponically grown leaves compared to soil grown leaves. In conclusion, our results confirm that plant responses present in hydroponic cultivation systems cannot be extrapolated straightforward towards soil grown plants. Therefore it is recommendable to include soil cultivation systems in studies with prospects to future field application.

Since – to our knowledge – until now no study has been conducted comparing the effects of hydroponic and soil cultivation system, this work offers novel insights in the effects of cultivation systems on plants proteomic response to metal stress.

Samenvatting

Atmosferische depositie afkomstig van zink fabrieken resulteerde in een grootschalige diffuse metaal vervuiling in NO België. Vermits toxische metalen zoals Cd, ernstige risico's kunnen vormen voor de menselijke gezondheid, werden er drempelwaarden opgelegd voor landbouwgrond en gewassen. Deze drempelwaarden worden echter vaak overschreden waardoor economisch waardevolle gronden verloren gaan. Hierdoor is de interesse van de overheid voor alternatieve remediatie technieken gestegen. Klassieke remediatie technieken zijn duur, tasten de bodemstructuur en micro-organismen aan en zijn niet toepasbaar voor grootschalige diffuse verontreinigingen. Fytoextractie, het *in situ* gebruik van planten om contaminanten te extraheren uit de vervuilde bodems, wordt gezien als een veelbelovend alternatief voor de zuivering van metaal gecontamineerde bodems. Om economisch aantrekkenlijk te zijn, moet de efficiëntie van dit proces echter verhoogd worden. Het uitrusten van plant endofyten met geschikte afbraakroutes, resulteerde in een verlaagde toxiciteit en evapotranspiratie van organische contaminanten. Dit succes wierp de vraag op of het uitrusten van plant endofyten met metaal sequestratie mechanismen een soorgelijk resultaat zou opleveren in metaal verontreinigde bodems.

In deel 3 werden 2 plantengroei bevorderende bacteriën uitgerust met het CZR operon, hetgeen ervoor zorgt dat bacteriën metalen kunnen binden op hun celwand. Gebaseerd op de onderzochte fenotypische eigenschappen leek de transconjugante stam W1366-5 het meest belovend. De resultaten van het serre experiment toonden echter aan dat transconjugante stam E1600-11 betere perspectieven biedt *in planta* wanneer deze blootgesteld zijn aan Cd (hoofdstuk 3.1).

Waargenomen verschillen in de effecten van Cd op de plant tussen hydro- en grondculturen leidden ertoe een gedetailleerde studie te verrichten naar de onderliggende mechanismen van Cd toxiciteit, gebruik makend van een proteomics benadering. Hiervoor werd de proteomics workflow eerst geoptimaliseerd (hoofdstuk 4.1). Vervolgens werd een literatuurstudie uitgevoerd om een beter overzicht te krijgen van de reeds gekende effecten van Cd op het proteoom van de plant; hierbij werd het duidelijk dat een vergelijking tussen proteoom studies niet zo eenvoudig is. De vastgestelde obstakels worden besproken in een meta-analyse (hoofdstuk 4.2). Hierin werd bovendien ook een vergelijking gemaakt tussen hydro- en grondcultuur experimenten wat duidelijk maakte dat planten verschillend lijken te reageren op Cd,

afhankelijk van de gebruikte cultuurmethode. De grote diversiteit aan groeiprotocols laat echter niet toe om deze hypothese hard te maken.

Om uit te maken of extrapolatie van hydrocultuur data naar grondcultuur experimenten mogelijk is, werd een experiment uitgevoerd waarin populier stekken gegroeid werden in hydrocultuur (hoofdstuk 4.3) en grondcultuur (hoofdstuk 4.4). Met uitzondering van het groeisysteem, werde andere parameters in beide experimenten gelijk gehouden. Het effect van Cd op populier werd onderzocht door de responsen van controle stekken te vergelijken met deze van Cd blootgestelde stekken na 3 en 21 dagen blootstelling.

Het vergelijken van de proteoom data van het hydrocultuur experiment (hoofdstuk 4.3) met deze verkregen uit de meta-analyse van in hydrocultuur gegroeide plant, brengt een algemene neerregulatie van CO_2-fixatie, chloroplast elektronen transport en ATP synthasen aan het licht in bladeren. Op het niveau van responsen van het stress metabolisme werden echter verschillen vastgesteld. In wortels werden in ons experiment dezelfde metabole pathways beïnvloed als diegene die naar voor kwamen uit de studie van de hydrocultuur data in de meta-analyse. Meer diepgaand onderzoek van de voorhanden zijnde gegevens bracht echter ook enkele verschillen aan het licht.

In de meta-analyse werd voor bladeren van planten gegroeid op bodems een algemene neerregulatie van CO_2-fixatie, chloroplast elektronen transport en ATP synthasen vastgesteld. Deze responsen werden echter niet bevestigd in onze studie (hoofdstuk 4.4). Net zoals voor de hydrocultuur studie werden in ons experiment weliswaar dezelfde metabole pathways beïnvloed als in de meta-analyse, maar werden bij een meer diepgaande studie van de resultaten ook verschillen gevonden.

Een vergelijking tussen beide experimenten (hoofdstuk 4.5) bevestigde de vermoedens dat planten gegroeid op hydrocultuur en op grond verschillend lijken te reageren op Cd. Het tijdseffect was duidelijk lager in hydrocultuur gegroeide bladeren vergeleken met bladeren gegroeid in grond. Deze data bevestigen dan ook dat responsen vastgesteld bij in hydrocultuur gegroeide planten niet zomaar geëxtrapoleerd mogen worden als representatief voor planten gegroeid op bodems.

Omdat er – voor zover wij weten – nog geen studies voorhanden zijn die de responsen van planten gegroeid in hydrocultuur en grond op Cd blootstelling vergelijken, levert dit werk nieuwe inzichten in de effecten van groeisystemen op de responsen van planten onder metaal stress.

Table of contents

Summary i

Samenvatting iii

Table of Contents v

List of Figures xi

List of Tables xv

List of Abbreviations xix

SECTION I: INTRODUCTION 25

CHAPTER 1.1: *Cadmium, a non-essential toxic metal* 27

1.1.1 General information 27

1.1.2 Historical pollution 28

1.1.3 Toxicological effects of Cd on humans 29

1.1.3.1 Exposure and localisation 30

1.1.3.2 Cellular responses of Cd 31

1.1.3.3 Diseases 33

1.1.3.4 Remedies for and prevention of Cd accumulation 34

CHAPTER 1.2: *Cadmium, responses in plants* 37

1.2.1 Uptake, transport and localization 37

1.2.2 Morphological and physiological effects 40

1.2.3 What happens within the cell upon Cd toxicity 42

1.2.4 Tolerance to Cd exposure 45

CHAPTER 1.3: *Approaches to cope with cadmium contaminated sites* 51

1.3.1 Which approach to choose? 51

1.3.2 Phytoremediation 53

1.3.2.1 General information 53

1.3.2.2 Phytoextraction of toxic metals 54

1.3.2.2.1 Hyperaccumulators versus high biomass producing plants 54

1.3.2.2.2 Phytoextraction efficiency determinants 55

1.3.3 Bacterial enhanced phytoextraction 56

1.3.3.1 Plant-associated bacteria 56

1.3.3.2 Engineered plant-associated bacteria 58

SECTION II: OBJECTIVES 63

SECTION III: BACTERIAL ENHANCED PHYTOEXTRACTION 67

CHAPTER 3.1: *Equipping plant growth-promoting bacteria (PGPBs) with a metal resistance systems: effects on metal uptake and translocation in poplar* 69

3.1.1 Introduction 69

3.1.2 Materials and methods 71

3.1.2.1 Conjugation 71

3.1.2.2 Phenotypical characteristics 72

3.1.2.2.1 Growth capacity on different media 72

3.1.2.2.2 Cd resistance 72

3.1.2.2.3 Plasmid stability 72

3.1.2.2.4 IAA production 73

3.1.2.2.5 Organic acid production 73

3.1.2.2.6 Siderophore production 73

3.1.2.2.7 ACC deaminase production 75

3.1.2.3 Genetic characterisation 75

3.1.2.3.1 BOX PCR 75

3.1.2.4 Cd extraction from liquid medium (Figure 3.1.1) 76

3.1.2.5 Cd extraction from contaminated soil 78

3.1.2.6 Plant cultivation 78

3.1.2.7 Inoculation of engineered PGPB 78

3.1.2.8 Statistical analysis 79

3.1.3 Results 79

3.1.3.1 Conjugation 79

3.1.3.2 Phenotypical and genotypical characterization 80

3.1.3.3 Cd extraction from liquid medium 84

3.1.3.4 Cd (im)mobilization from contaminated soil 86

3.1.3.5 Effects on poplar plants: a greenhouse experiment 87

3.1.4 Discussion 93

SECTION IV: PROTEOMICS 97

CHAPTER 4.1: *A comparative study of soluble protein extractions of Populus deltoides x (trichocarpa x deltoides) for two – dimensional gel electrophoresis* 99

4.1.1 Introduction 99

4.1.2 Materials and methods 100

4.1.2.1 Plant material 100

4.1.2.2 Extraction methods 101

4.1.2.2.1 TCA/acetone – phenol extraction 101

4.1.2.2.2 Acetone – phenol extraction 101

4.1.2.2.3 TCA/DTT/acetone extraction 102

4.1.2.2.4 A phenol extraction protocol rendering high resolution and reproducibility of 2-DE 102

4.1.2.3 RubisCO interference 102

4.1.2.4 Protein quantification 102

4.1.2.5 Two dimensional gel electrophoresis 103

4.1.2.6 Staining 104

4.1.2.7 Image analysis 104

4.1.2.8 Statistical analysis 104

4.1.2.9 Spot picking and identification 104

4.1.3 Results and discussion 105

4.1.4 Conclusion 112

CHAPTER 4.2: *Problems inherent to a metal analysis of proteomics data: A case study on the plants' response to Cd in different cultivation conditions* 115

4.2.1 Introduction 115

4.2.2 Diversity among experimental setup, material, methods and data analysis 117

4.2.2.1 Impediments with regard to cultivation methods and exposure 117
4.2.2.1.1 Cultivation methods: from the laboratory to the field 117
4.2.2.1.2 The applied light conditions, temperature and relative humidity 124
4.2.2.1.3 The applied Cd concentration 124
4.2.2.1.4 The chemical speciation of Cd 125
4.2.2.1.5 The applied exposure time 127
4.2.2.1.6 The studied tissues and organs 127
4.2.2.2 Impediments with regard to proteomics analysis 127
4.2.2.2.1 The diversity of material and methods peculiar to proteomics: from extraction to differential expression 127
4.2.2.2.2 The identification process 129
4.2.2.2.3 Statistics to determine differentially abundant proteins 132
4.2.2.2.4 The applied fold changes 132
4.2.3 Consensus in proteomics research... An illusion or plausible? 133
4.2.4 Validation and good reporting practice for protein identification 135
4.2.5 Guidelines for statistical analysis of differentially abundant proteins 137
4.2.6 Cadmium response – time and culture comparison 140
4.2.6.1 Time comparison 146
4.2.6.1.1 Hydroponic cultivation system – short term versus long term Cd exposure 146
4.2.6.1.1.1 Leaf proteome 146
4.2.6.1.1.2 Root proteome 149
4.2.6.1.2 Soil cultivation system – Short term versus long term cadmium exposure 151
4.2.6.1.2.1 *Root proteome* 151
4.2.6.2 Culture comparison 153
4.2.7 General conclusion 154
4.2.8 Perspectives 160
CHAPTER 4.3: *Effects of short and long term Cd exposure on the proteome of roots and leaves of hydroponically grown poplar (P. deltoides x (trichocarpa x deltoides))* 163
4.3.1 Introduction 163
4.3.2 Materials and methods 164
4.3.2.1 Plant cultivation: Hydroponic cultivation system 164

4.3.2.2 Analysis of metal content in plant tissue and hydroponic solution 166
4.3.2.2.1 Metal content in plant tissue 166
4.3.2.2.2 Metal content in hydroponic solution 166
4.3.2.2.3 Statistical analysis 166
4.3.2.3 Protein extraction 167
4.3.2.4 Protein quantification 167
4.3.2.5 DIGE labelling 167
4.3.2.6 2-DE 169
4.3.2.7 Analysis 169
4.3.2.8 Digestion and identification 170
4.3.3 Results and discussion 171
4.3.3.1 General effects 171
4.3.3.2 Effects of short and long term exposure to Cd on poplar 175
4.3.3.2.1 Effects on poplar leaves at proteome level 175
4.3.3.2.2 Effects on poplar roots at proteome level 183
4.3.4 Conclusion 192
CHAPTER 4.4: *Effects of short and long ter Cd exposure on soil grown poplar (P. deltoides x (trichocarpa x deltoides)) roots and leaves at proteome level* 197
4.4.1 Introduction 197
4.4.2 Material and methods 198
4.4.2.1 Plant cultivation 198
4.4.2.2 Analysis of metal content in plant tissue and soil 199
4.4.2.2.1 Plant available metal content in soil 199
4.4.2.2.2 Total metal content in soil 199
4.4.2.2.3 Plant metal content 199
4.4.2.2.4 Statistical analysis 200
4.4.2.3 Proteomic analysis 200
4.4.3 Results 200
4.4.3.1 General effects 200
4.4.3.2 Effects of short and long term exposure to Cd on poplar 204
4.4.3.2.1 Effects on poplar leaves at proteome level 204
4.4.3.2.2 Effects on poplar roots at proteome level 208

4.4.4 Conclusion 215

CHAPTER 4.5: *Effects of the cultivation system on the proteome of poplar (P. deltoides x (trichocarpa x deltoides)) leaves and roots* 219

4.5.1 Introduction 219

4.5.2 Effect of cultivation system on Cd content in leaves and roots 220

4.5.3 Effect of cultivation system on poplar proteome after Cd exposure 221

4.5.4 Effect of cultivation system on poplar proteome of control samples in time 224

4.5.5 Conclusion 226

SECTION V: GENERAL DISCUSSION, CONCLUSIONS AND PERSPECTIVES 229

5.1 Introduction 231

5.2 General discussion 232

5.3 Perspectives 237

SECTION VI: LITERATURE 241

SECTION VII: LIST OF PUBLICATIONS AND SCIENTIFIC MANIFESTATIONS 279

7.1 Scientific publications 281

7.2 Abstracts 282

7.2.1 Oral presentations 282

7.2.2 Poster presentations 283

7.3 Participation at scientific manifestations 285

List of Figures

Figure 1.1.1: Indirect Cd-induced generation of ROS. Source: Matovic *et al.*, 2011.

Figure 1.2.1: Cd caused cellular responses. Dashed lines: indirect effect, Full lines: direct effect, green: stimulating effect, orange: altered effect, red: inhibitory effect.

Figure 1.3.1: czc metal resistance mechanism. A three component metal export mechanism, consisting of CzcC, CzcB and CzcA proteins, is responsible for metal (M^{2+}) export. Due to the metal/proton antiport system, pH decreases, resulting in a metal precipitation on the bacterial cell wall. Two zones of regulatory genes, one upstream and one downstream, are involved in this bacterial defence system.

Figure 3.1.1: Cd extraction from liquid medium: schematic overview.

Figure 3.1.2: 1D gel-ectrophoresis analysis of BOX – PCR DNA fingerprinting. Loading scheme: 1 kb DNA ladder (GeneRuler™) – *Enterobacter* sp. 638 – E1600-11 – *P. putida* W619 – W1603-6 – W1366-5 – CM1600 – CM1601 – CM1602 – CM1603 – CM1366 - 1 kb DNA ladder (GeneRuler™).

Figure 3.1.3: Cadmium (µg) extracted from the medium after 2 days of bacterial growth. •—• indicates significant differences (two way ANOVA, $p<0.05$). No Cd was measured in Cd free media, independent of the bacterial strains and were therefore excluded from the graph.

Figure 3.1.4: pH difference measured in the medium after 4 days of bacterial growth. •—•indicates significant differences among the groups (two way ANOVA, $p<0.05$).

Figure 3.1.5 Cd (im)mobilization from contaminated soil. (A) Measured Cd concentration in the medium by ICP, (B) amount of Cd removed per bacterial unit; one bacterial unit represents $1*10^{-8}$ bacterial cells, (C) measured pH difference between time point zero and after 2 hours of contact with contaminated soil. Significant differences are indicated by letters, groups with the same letter are not significantly different (one way ANOVA, $\alpha <0.05$).

Figure 3.1.6: Effect of transconjugant strains on (A) weight (g) of leaves, shoot and root and on (B) shoot length (cm), root length (cm) and number of leaves. Data are presented as average ± standard error. Asterisks indicate significant differences with the non inoculated cuttings of the same exposure condition.

Figure 3.1.7: Effect of transconjugant strains on Cd concentration ($mg.kg^{-1}$ DW) in (A) leaves, (B) shoots and (C) roots. Data are presented as average ± standard error. Significant differences are present between control and Cd-exposed cuttings for all experimental groups. N.I.: non-inoculated.

Figure 3.1.8: (A) Amount of Cd (mg) removed by harvesting aboveground biomass and (B) amount of Cd (mg) retained in roots.

Figure 4.1.1: Comparison of the three extraction methods within the poplar leaves in a loop design; spots are revealed by silver staining. Extraction with TCA/acetone – phenol clearly rendered fewer spots and an inferior spot pattern. Therefore it was excluded from further analysis.

Figure 4.1.2: Comparison between acetone – phenol and TCA/DTT/acetone extraction; spots are revealed by silver staining. The acetone – phenol extraction (average total spot number of 983 ± 53, average background ratio of 0.905 ± 0.02) renders clearer spot patterns than the TCA/DTT/acetone extraction (average spot number of 606 ± 41, average background ratio of 0.759 ± 0.07)

Figure 4.1.3: Comparison of the extraction proposed by Sarma *et al.* (2008) and the acetone – phenol extraction; silver staining. Although a similar spot pattern was obtained using both extraction methods, the average total spot number was significantly higher with the acetone – phenol extraction (P = 0.0313). No significant differences were found between the average background ratio

Figure 4.1.4: Focusing of RuBisCO LS region. Spots are revealed by Gel Code Blue. A: 1.25% (v/v) IPG 4 – 7 NL buffer + 50 µg proteins loaded, B: 1.25% (v/v) IPG 4 – 7 NL buffer + 300 µg proteins loaded, C: no IPG 4 – 7 NL buffer + 300 µg loaded. By adding 1.25% (v/v) IPG 4 – 7 NL buffer the RuBisCO LS spot is clearly better focused

Figure 4.1.5: Acetone – phenol extraction of leaf samples. Gels were stained with Gel Code Blue and 300 µg proteins were loaded. After matching, a high reproducibility was present (Table I). The reference gel is shown with the spot pairs; red circles represent spots belonging to the reference gel (L12), blue circles represent spots belonging to gel L10. Numbered arrows indicate the spots that were picked for LCQ analysis.

Figure 4.1.6: Acetone – phenol extraction of poplar root samples; silver staining. This extraction method provides very clear spot patterns of soluble root proteins (average total spot number of 1611 ± 121 and an average background ratio of 1.03 ± 0.07)

Figure 4.2.1: Differences between cultivation methods considering controllability and their ability to resemble realistic conditions.

Figure 4.3.1: Hydroponic cultivation system. Water level was maintained by using the draining tube as a communicating vessel system; water was refilled bottom-up using a tube; air was applied using a glass aerating tube at the middle of the pot. Roots were kept in the dark at all times and were not manipulated during the experiment.

Figure 4.3.2: Effects of Cd on (A) weight of leaves, shoots and roots and on (B) shoot length, root length and number of leaves. Data presented as average ± standard error. Asterisks present significant differences (2 way ANOVA, $p<0.05$) between control and samples of exposed cutting of the same exposure period.

Figure 4.3.3: Metal concentrations in (A) poplar leaves, (B) poplar roots and (C) hydroponic solution. Data are presented as average ± standard error, b.d.l. = below detection limit. Asterisks present significant differences (2 way ANOVA, $p<0.05$) between control and samples of exposed cuttings of the same exposure period.

Figure 4.3.4: PCA analysis of 198 differentially abundant leaf proteins ($p<0.05$; two-way ANOVA). The left panel shows the distribution of spot maps regarding the two principal components (PC1 and PC2 explaining a cumulated 77.3% of all variation). The right panel shows the distribution of the proteins.

Figure 4.3.5: Hierarchical clustering of 198 differentially abundant leaf proteins ($p<0.05$, two-way ANOVA). Controls (day 3 + 21) and Cd-exposed groups after 3 days of exposure are grouped together. Cadmium-exposed group after 21 days of exposure is separated from the others.

Figure 4.3.6: PCA analysis of 601 differentially abundant root proteins ($p<0.05$; two-way ANOVA). The left panel shows the distribution of spot maps regarding the two principal components (PC1 and PC2 explaining a cumulated 54.7% of all variation). The right panel shows the distribution of the proteins.

Figure 4.3.7: Hierarchical clustering of 601 differentially abundant root proteins ($p<0.05$, two-way ANOVA). Controls (day 3 + 21) and Cd-exposed groups after 3 days of exposure are grouped together. Cadmium-exposed group after 21 days of exposure is separated from the others.

Figure 4.4.1: Soil cultivation system. Drainage system was composed of filter paper, 800 mg gravel (1 – 2 mm) and a cover to prevent roots to grow out of the soil compartment. Cuttings were planted in 4 kg sand and a cover was placed on top of the soil system to prevent algae growth. A dish was placed at the bottom to collect percolating water.

Figure 4.4.2: Effects of Cd exposure on (A) weight of leaves, shoots and roots and on (B) shoot length, root length and number of leaves. Data presented as average ± standard error. Asterisks presents significant differences (2 way ANOVA, $p<0.05$) between control and exposed cuttings of the same exposure period.

Figure 4.4.3: Metal concentrations in (A) poplar leaves and (B) roots. Data presented as average ± standard error, b.d.l. = below detection limit. Asterisks present significant differences (2 way ANOVA, $p<0.05$) between control and samples of exposed cuttings of the same exposure period.

Figure 4.4.4: PCA analysis of 779 differentially abundant leaf proteins (*p*<0.05; two-way ANOVA). The left panel shows the distribution of spot maps regarding the two principal components (PC1 and PC2 explaining a cumulated 68.3% of all variation). The right panel shows the distribution of the proteins.

Figure 4.4.5: Hierarchical clustering of 779 differentially abundant leaf proteins (*p*<0.05, two-way ANOVA). Control and Cd-exposed groups are grouped together according to the exposure period. A clear separation of the exposure periods is present.

Figure 4.4.6: PCA analysis of 121 differentially abundant root proteins (*p*<0.05; two-way ANOVA). The left panel shows the distribution of spot maps regarding the two principal components (PC1 and PC2 explaining a cumulated 74.2% of all variation). The right panel shows the distribution of the proteins.

Figure 4.4.7: Hierarchical clustering of 121 differentially abundant root proteins (*p*<0.05, two-way ANOVA). Control and Cd-exposed groups are grouped together according to the exposure period. A clear separation of the exposure periods is present.

List of Tables

Table 1.1.1: Properties and natural occurance of cadmium.

Table 3.1.1: Genotypical traits of *Enterobacter* sp. 638 and *Pseudomonas putida* W619. Genotypical traits derived from Taghavi *et al.*, 2009.

Table 3.1.2: Bacterial strains used in triparental conjugation.

Table 3.1.3: Growth capacity on different media. + indicated good bacterial growth, +/- indicates minor bacterial growth and – indicates no bacterial growth.

Table 3.1.4: Phenotypical traits of transconjugant strains; expressed in percentage of total. IAA: indole acetic acid, SID: siderophores, OA: organic acid.

Table 3.1.5: Phenotypical characteristics of parent strains and transconjugants selected for a greenhouse experiment. + : positive evaluation, - : negative evaluation, +/- : moderate positive evalutation, NT : not tested, IAA: indole acetic acid, SID: siderophores, OA: organic acid.

Table 4.1.1: Protein identification to determine RuBisCO interference.

Table 4.1.2: Matching results of the gel from the reproducibility test.

Table 4.2.1: List of articles studying cadmium response in plants. C.F.N.S. : Chemical form not specified, "?" represents no response on email about used statistics for differential expression.

Table 4.2.2: Light conditions applied in included research articles. If reported, temperature is specified as day/night temperature. N.S. = not specified.

Table 4.2.3: Distribution of articles included in the meta-analysis among different conditional groups.

Table 4.2.4: Number of differentially abundant proteins grouped per condition.

Table 4.2.5: Number of proteins grouped per cultivation system, exposure time and studied tissue.

Tabel 4.2.6: Total number of proteins in each conditional group per functional group. 'All proteins' represent the sum of all differentially abundant proteins per function from all conditional groups. Proteins of all conditions were considered together. Sum of subgroups is represented in bold. (N.S.SG = no specified subgroup). ABS represents the absolute number of proteins, percentage (Itallic) is calculated to the total differentially abundant (in- and decreased) proteins per conditional subgroup**.**

Table 4.2.7: Comparison to the working model proposed by Villiers *et al.* (2011). For each conditional subgroup, proteins involved in the working model of Villier *et al.* (2011) are shown to be increased (green) or decreased (red).

Table 4.3.1: Schematic overview of randomization of protein samples. In total, 10 leaf and root samples were labelled by CY3 and 10 leaf and root samples were labelled by CY5.

Table 4.3.2: List of identified proteins extracted from hydroponically grown leaves after 3 and 21 days of Cd exposure. CON: control, CAD: cadmium.

Table 4.3.3: Functional grouping of identified proteins extracted from hydroponically grown leaves after 3 and 21 days of Cd exposure. Absolute numbers (#) and percentages (%) are given. Percentages are calculated per conditional group.

Table 4.3.4: List of identified proteins extracted from hydroponically grown roots after 3 and 21 days of Cd exposure. CON: control, CAD: cadmium.

Table 4.3.5: Functional grouping of identified proteins extracted from hydroponically grown roots after 3 and 21 days of Cd exposure. Absolute numbers (#) and percentages (%) are given. Percentages are calculated per conditional group.

Table 4.4.1: List of identified proteins extracted from soil grown leaves after 3 and 21 days of Cd exposure. CON: control, CAD: cadmium.

Table 4.4.2: Functional grouping of identified proteins extracted from soil grown leaves after 3 and 21 days of Cd exposure. Absolute numbers (#) and percentages (%) are given. Percentages are calculated per conditional group.

Table 4.4.3: List of identified proteins extracted from soil grown roots after 3 and 21 days of Cd exposure. CON: control, CAD: cadmium.

Table 4.4.4: Functional grouping of identified proteins extracted from soil grown roots after 3 and 21 days of Cd exposure. Absolute numbers (#) and percentages (%) are given. Percentages are calculated per conditional group.

Table 4.5.1: Cd-induced growth reduction of hydroponically and soil grown poplar cuttings after 3 and 21 days of Cd exposure. Growth reduction was calculated as indicated above.

Table 4.5.2: Cd content (in mg kg^{-1} dry weigth) in leaves and roots of hydroponically and soil grown poplar cuttings after 3 and 21 days of Cd exposure.

Table 4.5.3: Functional grouping of differentially abundant, identified leaf and root proteins in both cultivation systems after 3 and 21 days of Cd exposure. Absolute numbers (#) and percentages (%) are given. Percentages are calculated per conditional group.

Table 4.5.4: Functional analysis of identified proteins, differentially abundant between control samples after 3 and 21 days of growth. Absolute numbers (#) and percentages (%) are given. Percentages are calculated per conditional group.

Table 4.1.1 (appendix): Spot number corresponds to the spots indicated in figure 1.5. Parameters obtained from BLAST against *Populus trichocarpa* v1.1 protein database (from left to right): the theoretical molecular weight (MW), protein identification number as entered in the database, number of unique peptides matched to obtain the protein sequence, number of unique spectra used in identification, the total number of spectra, the percent of coverage of the sequence obtained by mass spectrometry. Information obtained by search against NCBInr; *Populus* taxid 3689 database; (from left to right): the species to which the highest homology is found, the protein identification and percent homology found between the identified protein using *P. trichocarpa* v1.1 protein database and the identified protein using NCBInr; *Populus* taxid 3689 database (percent query coverage).

Table 4.3.2, 4.3.4, 4.4.1, 4.4.3 (appendix): Master No represents the spot number on the master gel. GI number represents the corresponding accession number in the NCBInr database. Protein name obtained via Blast2Go software by blasting against the NCBInr database. Appearance indicates the number of gels in which the spot appeares, the total gel number is given between brackets. 1-ANOVA represents the p-value with α = 0,05. Average ratio of the protein abundance is given for 3 and 21 days of exposure, positive values are gives as such, while negative values are given according to the following formula: given value = -1/average ratio.

SUPPLEMENTARY DATA

available at:
https://docs.google.com/file/d/0Bwj5DuhDKXzIYk1fdTFEX2tyb00/edit?usp=sharing

Table S1: List of all differentially abundant proteins listed by the selected articles for the meta analysis. GI number represents the corresponding accession number in the NCBI database (*viridiplantae*). Functional grouping was performed manually as described in 4.2.6.

Table S2: Detailed information considering protein identifications obtained from hydroponically grown leaves

Table S3: Detailed information considering protein identifications obtained from hydroponically grown roots

Table S4: Detailed information considering protein identifications obtained from soil grown leaves

Table S5: Detailed information considering protein identifications obtained from soil grown roots

Table S6: List of identified DAPs between control samples after 3 and 21 days of growth. Master No represents the spot number on the master gel. GI number

represents the corresponding accession number in the NCBInr database. Protein name obtained via Blast2Go software by blasting against the NCBInr database. Appearance indicates the number of gels in which the spot appeares, the total gel number is given between brackets. 1-ANOVA represents the p-value with *p*<0,05. Average ratio of the protein abundance is given for 3 and 21 days of growth, positive values are gives as such, while negative values are given according to the following formula: given value = -1/average ratio.

List of Abbreviations

2-DE	Two dimensional gel electrophoresis
AAS	Atomic absorption spectrometry
ABC	ATP-binding cassette
ACC	1-aminocyclopropane-1-carboxylic acid
Ala	Alanine
APX	Ascorbate peroxidise
As	Arsenic
Asa	Ascorbic acid
ATP	Adenosinetriphosphate
BF	Bioconcentration factor
BiP	Binding protein
BLAST(x/p)	Basic local alignment search tool (x: search protein database using a translated nucleotide / p: search protein database using a protein query)
BSA	Bovine serum albumine
CAT	Catalase
CAX	Calcium exchanger
Cd	Cadmium
CDF	Cation diffusion facilitator
CDI	Control deficit irrigation
CERCA	Comprehensive Environmental Response, Compensation and Liability Act
CFU	Colony forming unit
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
C_p	Metal concentration aboveground plant parts
C_r	Metal concentration root
C_s	Metal concentration shoot
C_{so}	Metal concentration soil
Cys	Cysteine
CZC	Cadmium zinc cobalt
CZR	Cadmium zinc resistance
Da	Dalton

DC	Detergent compatible
DAP	Differentially abundant protein
DHAR	Dehydroascorbate reductase
DIGE	Differential in gel electrophoresis
DTT	Dithiothreitol
DNA	Desoxyribonucleic acid
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
EF	Elongation factor
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
EST	Expressed sequence tag
FAD	Flavin adenine dinucleotide
FDH	Formaldehyde dehydrogenase
FDR	False discovery rate
FW	Fresh weight
GAS	General adaptation system
Glu	Glutamic acid
Gly	Glycine
Gln	Glutamine
GMO	Genetically Modified Organism
GSH-Px	Glutathione peroxidise
GST	Glutatione-S-transferase
GR	Glutathione reductase
GSSG	Glutathione (ox)
GSH	Glutathione (red)
HEPA	High-Efficiency Particulate Air
HMA	Heavy metal transporting P-type ATPase
HMT	Histone methyltransferase
HMW	High molecular weight
HSP	Heat shock protein
HUPO	Human proteome organization
IAA	Indole acetic acid
IARC	International Agency for Research on Cancer

ICP	Inductively coupled plasma
IEF	Isoelectric focusing
INPPO	International plant proteomics organization
IPG-NL	Immobilized pH gradient – Non linear
iTRAQ	Isobaric tag for relative and absolute quantification
kb	Kilobase
LC	Liquid chromatography
LCQ	Liquid chromatography quadrupole
LCT	Low affinity cation transporter
LOX	Lysyl oxidase
LS	Large subunit
M	Molarity
MAPK	Mitogen activated protein kinases
MDHAR	Monodehydroascorbate reductase
MIAPE	Minimum information about proteomics experiments
mRNA	Messenger ribonucleic acid
M_{rz}	Mass of soil volume rooted by the species under study
MS	Mass Spectrometry
MSENS	Multiple search engines, normalization and consensus
MT	Metallothioneins
MW	Molecular weight
N	Normality
NAD	Nicotinamide adenine dinucleotide (ox)
NADH	Nicotinamide adenine dinucleotide (red)
NE	North East
NL	Non linear
Nramp	Natural resistance-associated macrophage protein
NT	Not tested
OA	Organic acid
OD	Optical density
OEE	Oxygen-evolving enhancer protein
OES	Optical emission spectrometry
PAGE	Polyacrylamide gel electrophoresis
PC	Phytochelatin

PCA	Principal component analysis
PCBER	Phenylcoumaran benzylic ether reductase
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase
PGPB	Plant growth-promoting bacteria
PhR	Phytoextraction rate
pI	Isoelectric point
PMSF	Phenylmethylsulfonyl fluoride
PPO	Polyphenol oxidase
PR	Pathogen related
PRIDE	Proteomics identifications database
RC	Reducing agent compatible
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
rpm	Rotations per minute
RT	Room temperature
RuBisCO	Ribulose-1,5-bisphosphate carboxylase oxygenase
SDS	Sodiumdodecylsulfate
Ser	Serine
SID	Siderophore
SLC	Solute carrier family
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
TCE	Trichloroethylene
TF	Translocation factor
TI	Tolerance Index
TrF	Transcription factor
TRIS	Tris(hydroxymethyl)aminomethane
tRNA	Transfer ribonucleic acid
TSB	Tryptic soy broth
Ub	Ubiquitin
USP	Universal stress protein
UV	Ultra violet

V	Volt
v/v	Volume/volume
w/v	Weight/volume
w/w	Weight/weight
W	Watt
ZIP	ZRT, IRT-like protein

Section I:

INTRODUCTION

Chapter 1.1

Cadmium, a non-essential toxic metal

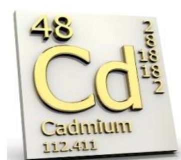
1.1.1 General information

In the 19th century, cadmium (Cd) was discovered as an impurity of zinc-carbonate (calamine). Cd is a soft, bluish-white transition metal with an atomic mass of 112.411 Da and the atomic number 48. Characterized by a $4d^{10}5S^2$ electron configuration, its most common oxidation state is Cd(II), however it also occurs as Cd(I). Cd is known as a toxic trace element with a density of 8.65 g cm^{-3} (Table 1.1.1). As a bulk metal, Cd is insoluble in water, resistant to corrosion and is not flammable. However, in powdered form, it may burn and release toxic fumes.

Cd is a natural occurring element, released naturally by among others, volcano eruptions, forest fires and erosion, with an abundance estimated at 0.1 – 0.5 µg g^{-1} in the earth's crust (Table 1.1.1). However, Cd levels in the environment vary widely, often depending on historical activities or natural occurring ores like zinc ores, which are mainly sulphides and oxides and typically contain the highest Cd levels (200 – 14,000 mg kg^{-1}).

Cd ranks 7th (out of 275) in a priority list of hazardous substances established by the US Comprehensive Environmental Response, Compensation and Liability Act (CERCA; ATSDR, 2007) and is classified as a human carcinogen by the International Agency for Research on Cancer (Waalkes, 2000). Unexpected, considering its toxic effects, a biological role for Cd has been discovered. In 2000, Lane and Morel proposed a functional role for Cd in the marine diatom *Thalassiosira weissflogii* under low zinc conditions. Under these conditions, *T. weissflogii* expressed a Cd-specific carbonic anhydrase. Since diatoms live in environments with very low zinc concentrations, their metabolism uses Cd rather than zinc. On top, according to Anke *et al.* (1987) Cd is an essential element in goat nutrition and goats require at minimum 50 µg Cd per kg DW a day. Cd and As deficit in goat's nutrition can have negative effects on growth and might cause health problems (Memisi *et al.*, 2008).

Table 1.1.1: Properties and natural occurrence of cadmium.



Properties		Natural occurence	
Symbol	Cd	Atmosphere	0.1 -5.0 ng m^{-3}
Atomic number	48		
Atomic weight	112.411	Earth's crust	0.1 - 0.5 µg g^{-1}
Group, period, block	12, 5, d		
Electron configuration	{Kr} 5s2 4d10	Marine sediment	1 µg g^{-1}
Density	8.65 g cm^{-3}		
Boiling point	767 °C	Sea water	0.1 µg l^{-1}
Melting point	321.07 °C		
Oxidative states	2, 1		

1.1.2 Historical pollution

From the late 19th century till the 70s of the 20th century, high amounts of Cd have been released into the environment due to the activity of zinc and nickel smelters. Due to this mobilization in the biosphere, the circulation of Cd through water, soil and atmosphere has increased tremendously (Nriagu and Pacyna, 1988). From 1970, electrolytic processes were used to extract zinc, resulting in a drop of annual Cd emissions from 125 000 kg in 1950 to 130 kg in 1980. However, since Cd is not degradable, this historical pollution is still present. In Belgium, more than 300 km² are classified as contaminated by heavy metals (MIRA 2007). Hot spots are located in the North-Eastern part of Belgium (Campine area), near old zinc smelters, resulting in loss of agricultural land and contaminated ground water. Next to industrial deposition, Cd is released in the environment by other anthropogenic activities such as application of pesticides, chemical fertilizers, waste water irrigation, sewage sludge, phosphate fertilisers, fossil fuel combustion, precipitation from heavy coal combustion and smelter wastes (di Toppi and Gabbrielli, 1999; DalCorso *et al.*, 2008; Gill and Tuteja, 2011; Matovic *et al.*, 2011). Industrial applications (batteries, pigments, corrosion protection, etc) were developed in the late 19th century. Cd emission

increased dramatically since Cd-containing products are rarely recycled (Jarup *et al.*, 2003).

As mentioned above, Cd is not very abundant in the earth's crust. In rock formations, Cd is mostly associated with a vast excess of zinc. Of the total Cd content in the soil, only a small fraction is available for plant uptake. The 'plant-available' Cd content is only in nanomolar range in most soils (Wagner, 1993). However, many factors such as soil pH and organic matter content strongly influence the size of the 'plant-available' fraction (Sauve *et al.*, 2000). The Campine area is characterized by sandy soils with a relative low pH, leading to a relatively high fraction of 'plant-available' Cd. As a consequence, Cd is easily taken up by plants, rendering this polluted area one of the main remediation sites in Belgium. Toxic metals that enter the plant roots pose a potential threat to human health. Accumulating in consumable parts of crop plants, toxic metals enter the human food chain. Since Cd belongs to the metals whose ions are most readily taken up by plant roots and can on top be translocated to aboveground plant parts, threshold values have been defined for agricultural soils and crops. Exceeding these threshold values and consequently loosing these economic valuable soils enhanced the authorities' interest in efficient remediation strategies. Classical remediation techniques (e.g. soil washing, excavation and dumping) are expensive, destructive for soil structure and microorganisms and not applicable for large scale contaminations. At present, in attendance of appropriate remediation strategies, cultivation strategies have been listed for several crops to maintain the Cd uptake under threshold values. Interest in using phytoextraction to clean up the widespread Cd contamination in the Campine area is growing since it seems a more and more promising strategy for the remediation of widespread metal contamination (see chapter 1.3).

1.1.3 Toxicological effects of Cd on humans

With a current European Cd intake close to the tolerable weekly intake, epidemiological studies on Cd toxicity concerning medical and public health implications are extremely important (Nawrot *et al.*, 2010).

1.1.3.1 Exposure and localisation

Exposure to Cd can be classified into two groups. The **first group** comprises daily environmental exposure arising from a variety of natural sources like polluted air (e.g. industrial polluted air, house dust and cigarette smoke) or consuming water and food (Luparello *et al.*, 2011). Cd is present in almost all kinds of food, with concentrations depending on the type of food and the level of soil contamination on which crops were cultivated (Matovic *et al.*, 2011). For the non-smoking population, food is the main source of Cd uptake (Cuypers *et al.*, 2010; Johri *et al.*, 2010). Worldwide estimates of dietary Cd intake range from 10-40 µg day^{-1} in non-polluted areas up to several hundred micrograms in Cd-polluted areas (Cuypers *et al.*, 2010). Adults across Europe are exposed to Cd levels nearing or slightly exceeding the tolerable weekly intake of 2.5 µg kg^{-1} body weight (Nawrot *et al.*, 2010; Matovic *et al.*, 2011). For some subgroups of the population, the data is even more dramatic: vegetarians, women in the reproductive phase of life, people living in highly contaminated areas and smokers may exceed the tolerable weekly intake by about 2-fold (Nawrot *et al.*, 2010). Next to food, cigarette smoke is the main source of non-occupational exposure. Heavy smokers (smoking more than one cigarette pack a day) have a Cd body content twice as high as non-smoking persons (Matovic *et al.*, 2011). The **second group** comprises occupational exposures found in industries such as electroplating, smelting and refining, welding, battery manufacturing and pigment production (Nawrot *et al.*, 2010).

The routes of Cd intake involve lungs, intestines and in low amounts the skin. In the body, Cd is predominantly bound to metallothioneins (Hamer, 1986). The Cd-metallothionein complex is distributed to various tissues and organs, but is ultimately reabsorbed in kidney tubuli (Ohta and Cherian, 1991). According to the study of Horky *et al.* (2002), Cd is rapidly absorbed by the small intestine mucosa and subsequently transported to liver and kidneys, where it is deposited. Reduced silver granules, representing heavy metals, were present in nearly all of the distal and proximal tubules of the nephron. Having a half-life of 20-35 years, Cd accumulates in human tissues, with the highest concentrations measured in the kidneys, liver, pancreas, lungs and testes (Nawrot *et al.*, 2010; Jomova and Valko, 2011). In order to cause toxic cellular responses, Cd must first enter the cells. Reviews on Cd transport in mammalian cells have recently

been published (Thévenod, 2010; Van Kerkhove *et al.*, 2010; Vesey, 2010). The localisation of Cd in the cell is highly tissue-dependent. Moreover, the subcellular localisation depends on the presence of other elements like Zn and Se (Horky *et al.*, 2002). To our knowledge, there is no paper published concerning the subcellular Cd localisation in humans.

1.1.3.2 Cellular responses of Cd

Although substantial efforts have been made to clarify the mechanisms of cellular Cd toxicity in human cells, the precise mechanisms remain unclear (Matovic *et al.*, 2011).

As a redox inactive metal, Cd itself is unable to directly generate **oxidative stress** through the production of free radicals. However, Cd can generate reactive oxygen/nitrogen species (ROS/RNS) indirectly (Waisberg *et al.*, 2003) by (1) impairing enzyme activity of antioxidative defence system (superoxide dismutase; SOD, catalase; CAT, glutathione peroxidase; GSH-Px, glutathione-S-transferase; GST, glutathione reductase; GR), by (2) disturbing the generation of the non-enzymatic component glutathione; GSSG and GSH and by (3) elevating the levels of Fenton active metals (Fe^{3+} and Cu^{2+}) (Figure 1.1.1) (Ahsan *et al.*, 2009; Matovic *et al.*, 2011). Cd toxicity caused by interference with the homeostasis of essential metal cations in animal cells is reviewed by Moulis (2010). Therein the interaction between Cd and zinc, iron, manganese and copper is discussed. Upon long term exposure to environmental Cd, oxidative stress can be the result of dysfunction of mitochondria (Gobe and Crane, 2010).

Cd toxicity generates **genetic instability** by inducing different types of DNA damage. The number of cells with DNA single strand breaks and the levels of cellular DNA damage were significantly higher in Cd-exposed groups than in controls (Yang *et al.*, 2003). DNA can be damaged through Cd-induced ROS. Nevertheless, oxidative DNA damage does not appear to be sufficient to explain the toxic effects of Cd (Hartwig, 2010). A more important cause of Cd-induced DNA damage is the impairment of almost all major DNA repair pathways. Multiple evidence is present indicating Cd interference with nucleotide excision repair, base excision repair and mismatch repair (for an in-detail review, we refer to Hartwig, 2010).

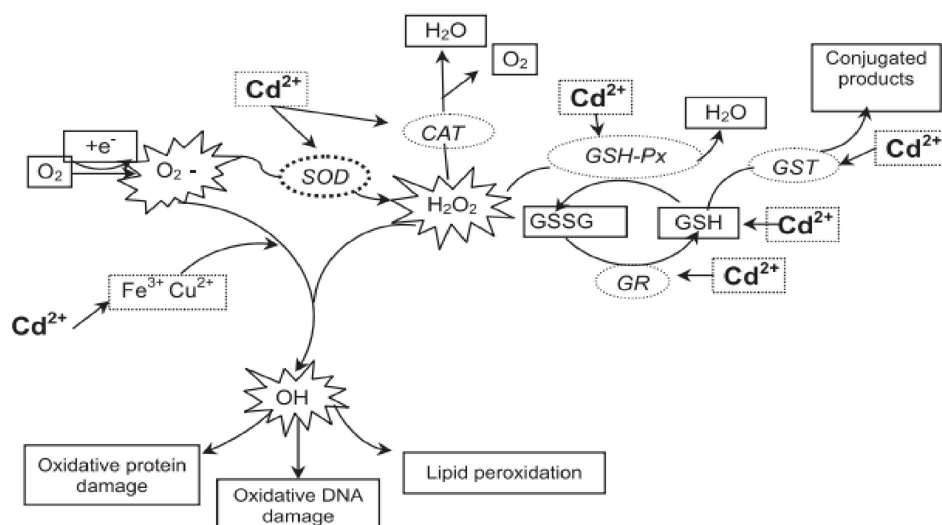


Figure 1.1.1: Indirect Cd-induced generation of ROS. Source: Matovic *et al.*, 2011.

In addition to its interference with DNA repair systems, Cd also interferes with DNA damage report systems. By disturbing the function of the tumor suppressor protein p53, Cd interferes with the **cell cycle control** in response to DNA damage (Meplan *et al.*, 1999). Furthermore, human prostate epithelial cells acquired resistance to **apoptosis** upon Cd exposure. Consequently, by escaping the elimination procedure through apoptosis, damaged cells could enter the cell cycle, allowing them to replicate damaged DNA with high frequency of mutations, which may play an important role in Cd-induced carcinogenicity (Hartwig, 2010). Moreover, **gene expression** is altered for numerous genes upon Cd exposure. The mainly affected genes can be classified into four groups: (1) major stress response genes, (2) immediately early response genes, (3) transcription factors and (4) translation factors (Hartwig, 2010).

Many of the biological effects may be due to the interaction, often antagonistic, between Cd and essential elements (Lazarus, 2010; Hartwig, 2010). As stated above, Cd can replace iron and copper in various cytoplasmic and membrane proteins, thereby increasing the amount of unbound free or poorly chelated copper and iron ions that can participate in oxidative stress via the Fenton reactions (Price and Joshi, 1983). At the same time, conformational changes

occur upon replacement of the coordinated metal, resulting in damage and loss of function of different biomolecules (Jomova and Valko, 2011). Although it is known for a while that Cd interferes with the biokinetics and biological roles of many essential metals and metalloids and it reduces the levels of essential elements, the exact underpinning mechanisms are not yet entirely identified. Competing for the same binding site with Zn, Cd-induced disturbances in Zn homeostasis may have severe consequences on cell growth, development and function (Matovic *et al.*, 2011). Finally it should be mentioned that Cd may cause numerous cytotoxic and metabolic effects that have not been sufficiently recognised (Matovic *et al.*, 2011).

1.1.3.3 Diseases

Although Cd is now known as a hazardous element, it was applied in medicine science in the early 20th century. Even in the past decade, Cd-containing nano-particles had numerous biomedical applications, especially in the diagnosis of cancer. Not surprising, Cd-containing quantum dots are potentially toxic and their use involves substantial risks (Matovic *et al.*, 2011).

Since its use, diverse symptoms have been described related to Cd, affecting numerous organs including kidneys, liver, lungs, pancreas, testes and bones. Forty years after its discovery, acute gastrointestinal and respiratory symptoms were reported. Later on, due to increased occupational exposure, toxic effects of Cd on the lungs, kidneys and bones were confirmed. Most public attention arose when the itai-itai disease, a bone disease characterised by fractures and severe pain, was linked to Cd (Matovic *et al.*, 2011). On top, Cd is a potent human carcinogen (classified as a human carcinogen category I by the International Agency for Research on Cancer (IARC) and the US National Toxicology Program) causing preferentially lung and gastro-intestinal (kidney and pancreas) cancers (Jomova and Valko, 2011). Reviewing the health risks due to Cd exposure, Nawrot *et al.* (2010) grouped the effects on humans into: osteoporosis, kidney-related damage, diabetes, cancer, effects on blood pressure and arteries and finally effects on reproduction and mortality. We refer to their review for more in depth information on this topic.

Many of the DNA base modifications caused by free radicals are pro-mutagenic, pointing to a strong link between oxidative damage and the carcinogenesis of

metals (Jomova and Valko, 2011). However, current research suggests that no direct genotoxicity but rather multiple indirect mechanisms are operative for tumor induction. The underlying mechanisms comprise (1) increased oxidative stress, (2) interactions with the cellular DNA damage response systems, including DNA repair processes, cell cycle control and apoptosis as well as (3) epigenetic changes in DNA methylation patterns, leading to a high degree of genomic instability (reviewed by Hartwig, 2010).

1.1.3.4 Remedies for and prevention of Cd accumulation

Prevention of Cd accumulation in the body can be performed at multiple levels. One way is to **control the 'bio-availability' of Cd in the soil and its transfer to plants**, especially consumable plants. The capacity to absorb and accumulate Cd varies extensively between plant species and varieties. Generally, 'leaf vegetables' tend to accumulate relatively high concentrations of Cd, whereas 'fruit vegetables' accumulate only low concentrations of Cd (Nawrot *et al.*, 2010). Therefore, guidelines have been developed, describing how to cultivate vegetables on Cd contaminated agricultural soils. Unlike agriculturally grown vegetables, home grown vegetables are not subjected to quality control. In the cadmium contaminated part of the Campine area, the Cd content in several home grown vegetables exceeds the European limit (Nawrot *et al.*, 2010). Therefore 'home farmers' are advised to take a representative soil sample to determine the soil Cd concentrations. On top, soil pH should also be controlled (maintained close to neutral: pH-H_20 of 7.5; pH-KCl of 6.5) since it has a great influence on the 'plant availability' of Cd. To **lower the amount of Cd-polluted dust particles** in the atmosphere, generally generated by wind taking up polluted soil particles, a well-closed cover of plants could be installed on the contaminated soils. In-house prevention can be achieved by replacing carpets by floor coverings and by using a vacuum cleaner with a HEPA-filter, combined with cyclone technology to remove particles as small as 0.3 μm. Another way to prevent Cd accumulation in the body is to **decrease its intestinal absorption**. When dietary essential metals are in short supply and deficiencies develop, Cd absorption and toxicity are enhanced (Nawrot *et al.*, 2010; Vesey, 2010). Under iron deficiency, the duodenal iron transporter is up-regulated, leading to an increased intestinal absorption of dietary Cd (Nawrot *et al.*, 2010). Since iron

deficiency is considered to be one of the main nutritional deficiency disorders affecting large parts of the European population, this should be addressed as a main issue of concern for human health. In this prospect, exposed persons are suggested to include essential nutrient supplementation in everyday diet (Lazarus, 2010; Matovic *et al.*, 2011). Dietary zinc supplementation may prevent Cd-induced disorders in bone turnover and could have a beneficial effect on Cd-induced carcinogenesis. Magnesium has the ability to protect the kidney from Cd accumulation and has beneficial effects against Cd-altered renal copper and zinc levels (Matovic *et al.*, 2011). Although zinc reduced Cd tissue levels more efficiently, magnesium is proposed as the dietary supplement of choice for the reduction of Cd body burden, since addition of magnesium did not alter zinc or copper levels. As a constituent of selenoproteins, proteins with anti-oxidant properties that bind Cd, selenium seems interesting as well (Lazarus, 2010). On top, vitamin E and/or vitamin C seem to display a protective role against the toxic effects of Cd (Jomova and Valko, 2011).

Chapter 1.2

Cadmium responses in plants

Being sessile organisms, plants need to cope with the ever changing environment surrounding them. *In vivo* plant responses to Cd depend on (1) the plant species/subspecies/variety/ecotype (e.g. hyperaccumulators or genetically resistant/tolerant plants will respond differently), (2) duration of Cd exposure and (3) external environmental conditions which can alter the plants' sensitivity to Cd. This subsection will focus on the responses to Cd *in planta*, however it must be mentioned that part of this response belongs to a common plant response under conditions of an excess of toxic non-essential metal ions.

1.2.1 Uptake, transport and localization

The rate at which higher plants take up Cd depends on its concentration in the soil and its 'availability' to plants. The plant 'availability', defined as 'the part of the total concentration of a chemical that is available to plants' is mainly influenced by the soil pH. Other factors such as the presence of organic matter, the redox potential, the temperature, the rhizosphere (root exudates and the presence of mycorrhiza, rhizospheric bacteria), cultivar, age of the plant and the concentrations of other elements might have significant effects as well (di Toppi and Gabbrielli, 1999; DalCorso *et al.*, 2008; Sarwar *et al.*, 2010). To enhance soluble metals in the soil, plants might excrete ligands such as organic acids, histidine, phytosiderophores and nicotianamine (Callahan *et al.*, 2006). Exudation of carboxylase into the rhizosphere is also considered to be able to enhance metal accumulation in plants (Clemens *et al.*, 2002). Most Cd present in the plant is taken up through the roots, only a small part is taken up from the atmosphere (Clemens, 2006b).

Mechanisms that control the uptake of Cd by plant roots and those regulating the accumulation in the edible parts of the plant are not well understood yet (Hasan *et al.*, 2009) and vary considerably between plant species. Cd can be absorbed as inorganic complexes (e.g. $CdCl^+$, $CdCl_2$, $CdSO_4$) or organic complexes (e.g. PC-complexes, MT-complexes) (McLaughlin *et al.*, 1996). Due

to their negative charge, cell walls have a significant capacity for binding metals, resulting in an increased metal concentration compared to that of the cytoplasm (DalCorso *et al.*, 2010). A driving force for the uptake of cations is provided by membrane potential and intracellular metal binding or sequestration sites (Benavides *et al.*, 2005; Clemens, 2006b). The large negative membrane potential alone provides more than enough energy to drive Cd^{2+} uptake even at low concentrations (Hasan *et al.*, 2009). At the root surface, Cd competes with other cations to gain access into the plant cell via transport mechanisms for micronutrient uptake. There is clear, although mostly indirect, evidence that Cd can enter the plant roots via Ca^{2+}, Fe^{2+} and Zn^{2+} transporters/channels (Clemens, 2006b; Van Belleghem *et al.*, 2007). Further, there is an indication of the existence of a Cd^{2+}-specific uptake system in *T. caerulescens* (Clemens *et al.*, 2006b) and an energy dependent transmembrane transport of $CdCl_2$ has been reported as well (Heber *et al.*, 2002). Most Cd ions remain at the root level, however after reaching the xylem and/or phloem, they can be transported to the aboveground tissues following the water stream (di Toppi and Gabbrielli, 1999, DalCorso *et al.*, 2008). Efficient translocation of metal ions to the shoot requires radial symplastic passage, an active loading into the xylem and the availability of suitable ligands in the xylem (Clemens *et al.*, 2002). Characterized by high mobility and high water solubility, Cd can easily enter the roots cortical tissues by passive diffusion (Van Belleghem *et al.*, 2007). Subsequently, complexed by several ligands, Cd can reach the xylem through apoplastic and/or symplastic transport. Studying X-ray absorption data revealed that Cd is chelated by thiolates in root cells but by nitrogen and oxygen ligands in the xylem (Salt *et al.*, 1995). Cataldo *et al.* (1983) stated that Cd is primarily associated with components of the amino acid/peptide fraction of soybean xylem sap. Although there is no direct evidence for Cd complexes in the phloem sap, they are believed to bind to ligands like nicotianamine, metallothioneins and phytochelatins. Additionally, indirect evidence for a participation of phytochelatins in translocation of Cd has been reported by Gong *et al.* (2003). The lack of energy for Cd sequestration and the Cd-induced cellular damage in roots may be driving factors for its translocation from roots to aerial plant parts (Sarwar *et al.*, 2010). The P1B-type ATPase AtHMA4 is the first identified transporter involved in Cd translocation from root to shoot (Verret *et al.*, 2004;

Mills *et al.*, 2005). HMAs (heavy metal transporting P-type ATPases) are pointed out as good candidates to transport metal ions and/or metal ion complexes across the cell membrane (Axelsen and Palmgren, 2001). Unfortunately, based on the scarcity of available data, no mechanisms can yet be proposed for Cd translocation. Although less reported, the same pathways for sequestration and metal uptake are believed to be addressed in leaf cells (Clemens, 2006b).

Considering the partitioning of Cd between tissues, well-documented differences among species are reported (Wagner *et al.*, 1993). Accumulation, as well as distribution of Cd throughout the plant differs depending on the species, ecotype and growing conditions. On top, both are influenced by the presence of other elements (Grant *et al.*, 2008). Distribution of Cd within the plant is influenced by transport from roots to the shoots via the xylem, transfer from the xylem to the phloem and transport through the phloem from sources to sinks (Riesen and Feller, 2005). Cd has a higher propensity to accumulate in tissues other than the roots, compared to other toxic metals or metalloids (Clemens, 2006b). Four factors are proposed to determine Cd partitioning in plants: (1) the activity of metal-sequestering pathways in root cells, which is likely to play a key role in determining the rate of translocation to the aerial parts, (2) the degree of accessibility and mobilization of sequestered metal ions, (3) the efficiency of radial symplastic passage through the root and across the endodermis and (4) the xylem loading activity. In general, the concentration of Cd in plants decreases in the order: root > leaves > fruits > seeds (Sharma *et al.*, 2006). Depending on the species, Cd was localized in the leaf vacuoles, cell wall, epidermis and/or mesophyll (Cosio *et al.*, 2005) and in the tracheids (Van Belleghem *et al.*, 2007). A study on leaves of *T. caerulescens* indicated that tissues could be ranked with respect to the Cd concentration as follows: apoplasm/cell walls > lower epidermis > mesophyll protoplast (Cosio *et al.*, 2005). Interestingly, the same study indicated that Cd distribution patterns can change with increasing Cd concentration applied, since they observed a Cd shift from young to old leaves when the concentration increased. The same is described in *Arabidopsis thaliana* plants exposed to Cd (Van Belleghem *et al.*, 2006). Precise subcellular localisation of Cd at high concentrations is however hindered due to plasmolytic shrinkage (Van Belleghem *et al.*, 2007). Therefore it is suggested that subcellular localisation of Cd should be done on lower Cd

concentrations that do not induce detrimental plasmolytic shrinkage. The duration of the Cd exposure does not appear to influence the Cd distribution pattern (Cosio *et al.*, 2005). Taken together, these statements illustrate the difficulty of interpreting Cd subcellular localization data.

1.2.2 Morphological and physiological effects

Once taken up by the plant, Cd induces different toxic effects. At morphological level stunted shoot growth, chlorosis, leaf epinasty and root reduction are present. At high exposure, anthocyanin-coloring in *Arabidopsis* leaves was described (Van Belleghem *et al.*, 2007). On top, a recent study indicated changed root architecture upon Cd exposure (Remans *et al.*, 2012). The alterations in normal plant growth are mainly due to interference with photosynthesis, respiration, water relations and with the uptake and translocation process of minerals (DalCorso *et al.*, 2008; Gill and Tuteja, 2011). It is however clear that Cd toxicity depends on the exposure time, the concentration to which plants are exposed and the studied plant species (Smeets *et al.*, 2005).

A variety of physiological/biochemical/molecular effects lay at the base of the above described morphological effects. **At first**, several effects caused by Cd exposure are linked to an (directly or indirectly) altered uptake of water. Nutrient metabolism (uptake, transport and use) is affected as well: by (1) interfering with the uptake of Ca, Mg, K and P (Benavides *et al.*, 2005 in DalCorso 2008), by (2) inhibiting the nitrate reductase activity in the shoots, Cd reduces the absorption of nitrate and its transport to the shoots (Hernandez *et al.*, 1996; Gill and Tuteja, 2011), and by (3) the inhibition of root Fe(III) reductase which leads to Fe(II) deficiency and consequently affects photosynthesis (Alcántara *et al.*, 1994). On top, the presence of Cd in the soil leads to uptake competition with other elements which can result in deficiencies of trace elements (Benavides *et al.*, 2005). **Secondly**, Cd interacts with several cellular activities like water balance (Clemens, 2006a), photosynthetic activity (pigment content, non-photochemical quenching, disruption of the photosynthetic apparatus; Sanità di Toppi and Gabbrielli, 1999), protein synthesis (Dalcorso *et al.*, 2008) and oxidative mitochondrial phosphorylation (Keunen *et al.*, 2011). On top, Cd greatly impedes ion homeostasis, resulting in

stomatal closure, chlorosis and increased ethylene levels (Sanità di Toppi and Gabbrielli, 1999; Clemens, 2006a). Stomatal closure during Cd exposure is independent of the water status; it is caused by interference of Cd with movements of K^+, Ca^{2+} and abscisic acid in the guard cells (MacRobiie and Kurup, 2007; Perfus-Barbeoch *et al.*, 2002). Similarly, chlorosis is considered to be a result of Cd-induced changes in Fe : Zn ratios (Root *et al.*, 1975). Finally, Cd generated perturbations in Ca levels could interact with and stimulate ethylene production (Rodríguez-Serrano *et al.*, 2006, 2009; Arteca and Arteca, 2007). **Thirdly**, many cellular pathways are altered or disrupted upon Cd exposure. The presence of Cd has an effect on expression of several genes and strongly affects the activity of several enzymes (CO_2-fixation, Calvin cycle, carbohydrate metabolism, phosphorus metabolism, nitrogen and sulphur metabolism and the primary ammonia assimilation) (di Toppi and Gabrielli, 1999; Gill and Tuteja, 2011). Since Cd is a non redox-active metal, it is not taking part in the Haber-Weiss and Fenton reactions. The symptoms of oxidative stress are a consequence of the GSH depletion due to binding of Cd to GSH and to formation of GSH-derived phytochelatins (Clemens, 2006; Cuypers *et al.* 2011). The presence of Cd induces a general redox homeostasis impairment (reviewed by Cuypers *et al.*, 2011), triggering H_2O_2 and O_2^- over-accumulation (DalCorso *et al.*, 2008). Subsequently, enzymes linked to oxidative stress and the anti-oxidative defence mechanisms are well known enzymes which activity is altered due to Cd toxicity (Smeets *et al.*, 2005; Smeets *et al.*, 2009; Cuypers *et al.*, 2011). Next to enzymatic responses, Cd is known to enhance ROS production in subcellular organelles as a result of their highly oxidizing metabolic nature and the presence of electron transport chains (Halliwell, 2006). For a review on metal induced ROS production in mitochondria, we refer to Keunen *et al.* (2011). However, at present it is not yet clear if overproduction of ROS is the cause of redox cellular imbalance or if this imbalance is a specific plant cell strategy to cope with metal stress (Schützendübel *et al.*, 2002a; DalCorso *et al.*, 2008). On top, a secondary effect caused by the Cd-induced ROS accumulation is the alteration of signalling mediated by H_2O_2 and other oxygen species (DalCorso *et al.*, 2008). The above described effects on genes and proteins are generally due to (1) the high affinity of Cd for sulphydryl groups of structural proteins and enzymes which leads to protein misfolding, inhibition of activity

and/or interference with redox enzymatic regulation, (2) Cd-induced post-translational changes (carboxylation and oxidation of sulphur-containing amino acids) and (3) the chemical similarity between Cd^{2+} and functionally active ions situated in active sites of enzymes and signalling molecules (Hall, 2002; Villiers *et al.*, 2011). **Finally**, Cd can have an effect on host – pathogen interactions (Aloui *et al.*, 2009; Farinati *et al.*, 2009; Ghoshroy *et al.*, 1998; Repetto *et al.*, 2003; Stroinski, 1997) and lately it was shown that the presence of Cd alters the endophytic bacterial community (phenotypically) in *Brassica napus* L. (Croes S, personal communication, unpublished data) and in seeds of *Arabidopsis thaliana* (Truyens *et al.*, 2012).

1.2.3 What happens within the cell upon Cd toxicity

Before any morphological effects appear, toxic internal Cd concentrations are interfering at molecular and biochemical levels (Lagriffoul *et al.*, 1998, Smeets *et al.*, 2005; Cuypers *et al.*, 2011). Mitochondria as well as plasma membranes are considered as primary cellular targets of Cd (Vangronsveld and Clijsters, 1994; Cuypers *et al.*, 2009; Cuypers *et al.*, 2010). Once in the cell, Cd disrupts the overall metabolism in numerous ways and on several levels, making the response to Cd in higher plants a complex phenomenon (Figure 1.1.2). The complexity is believed to be dependent on the intensity of the stressor (Cuypers *et al.*, 2011). In plants, the presence of Cd leads to altered signalling within the cell. This is caused by (1) a perturbation of intracellular calcium level and interference with the calcium signalling, through substituting Ca^{2+} in calmodulin regulation, (2) an up-regulation of Mitogen-Activated Protein Kinases (MAPKs), (3) the regulation of plant hormone synthesis and (4) via H_2O_2 production (reviewed by DalCorso *et al.*, 2010). MAPKs make up evolutionary conserved signal transduction cascades that convert extracellular signals to appropriate cellular responses (Opdenakker *et al.*, 2012). Moreover, it is suggested that LOX genes, up-regulated upon cadmium exposure might have a role in root-to-shoot signalling via the production of oxylipines and jasmonates (Smeets *et al.*, 2009). Up to date it is not clear whether the first two signalling pathways are a direct result of Cd or an indirect result due to Cd-induced ROS (Deckert, 2005). Signal sensing and cellular redox imbalance are strongly dependent on the studied plant organ and the chemical properties of the metal (Cuypers *et al.*, 2011).

Intracellular signalling targets numerous transcription factors (TrF), resulting in an altered gene regulation. By replacing cations in a variety of catalytic sites and due to its high affinity for cysteine, glutamate, aspartate and histidine, Cd induces an altered gene regulation and altered enzyme activities (Vangronsveld and Clijsters, 1994; Waalkes, 2000). ROS induce the formation of disulphide bridges, protein – protein crosslinks and are responsible for the oxidation of amino acid side chains and protein backbones. As a result, Cd indirectly induces protein fragmentation and subsequently proteolytic activity (Berlett and stadtman, 1997). Not only proteins are targets of Cd-induced damage; the presence of Cd induces DNA damage as well. Different ways (direct or indirect) are addressed: (1) inducing changes in DNA-synthesizing enzymes, (2) influencing DNA repair processes, (3) binding of Cd^{2+} to DNA, (4) targeting DNA-binding proteins, (5) cation substitution in a variety of catalytic sites, (6) inhibition of mismatch repair and (7) ROS production (Deckert, 2005). Due to DNA damage, Cd alters the cell cycle at the G1/S checkpoint. On top, it can alter the cell cycle at the G2/M checkpoint as well, due to a Cd-induced decrease of cyclin B1 mRNA (Sobkowiak and Deckert, 2003; 2004). These events ultimately lead to an altered cell division. So far, telomerase is the only enzyme described to be involved in repair of Cd-induced DNA damage in plants (Fojtova *et al.*, 2002). Additionally, an increased RNA content is present, mediated indirectly by a Cd-induced decrease in RNAse activity (Hirt *et al.*, 1989; Shah and Dubey, 1995).

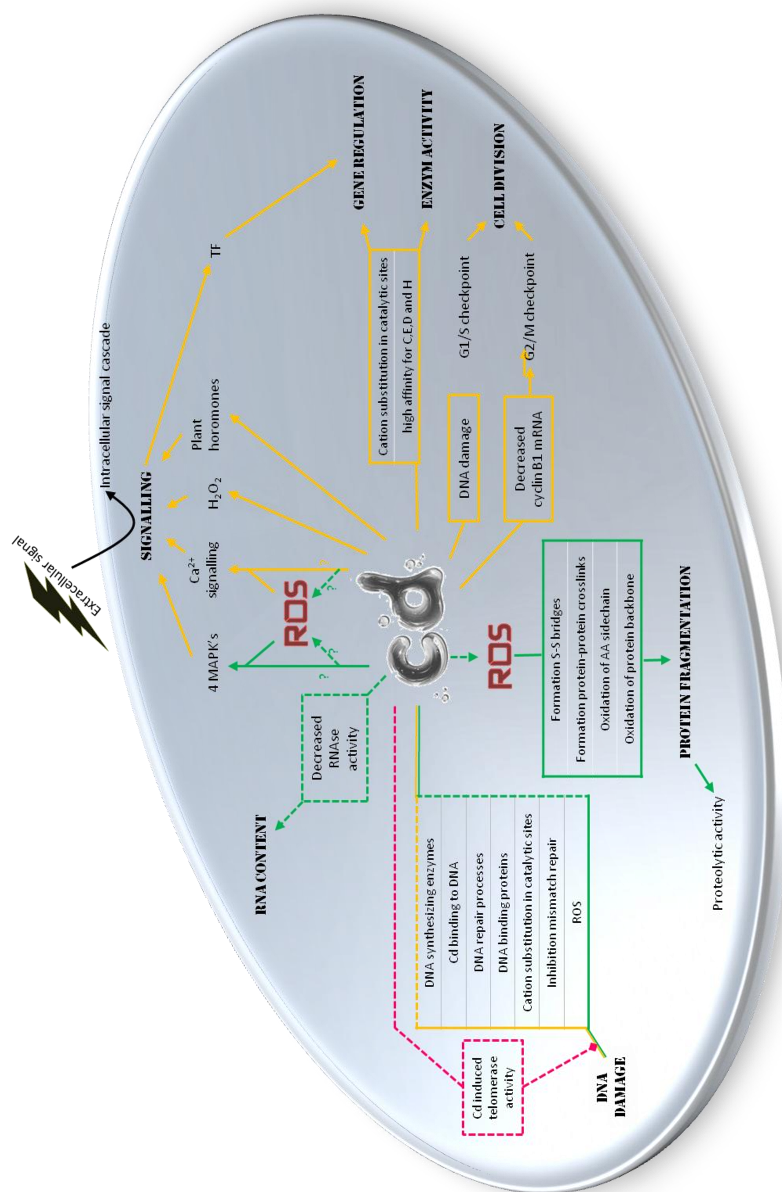


Figure 1.2.1: Cd caused cellular responses. Dashed lines: indirect effect, Full lines: direct effect, green: stimulating effect, orange: altered effect, red: inhibitory effect.

1.2.4 Tolerance to Cd exposure

By changing gene expression, altering cell cycle and division, disrupting proteins and nucleic acids, affecting cellular signalling and altering enzyme activities, Cd rapidly brings the cell in a state of toxicity. A decreased effect of Cd over time was described in a poplar study (Kieffer *et al.*, 2009a), suggesting a general protective response of plants in order to lower the effects of Cd and survive metal exposure (Villiers *et al.*, 2011). Apparently all plants possess some basic tolerance to toxic non-essential metals. As a response to Cd toxicity, the plant cell is equipped with a number of defence mechanisms. Although exact borderlines are hard to premise, the defence mechanisms can be divided into 'first line' and 'second line' defence mechanisms; it is note-worthy to mention that a 'fan-shaped model' is proposed as an alternative (di Toppi and Gabrielli, 1999). The **first line defence mechanisms** include (1) exclusion, (2) immobilisation, (3) compartmentalisation, (4) synthesis of phytochelatins and (5) synthesis of metallothioneins. These mechanisms could be considered as mechanisms to prevent Cd of inducing cell stress. However, when the first line defence mechanisms are insufficient to cope with the excess of metals and damage is occurring, **second line defence mechanisms** are addressed, including (6) synthesis of stress-related proteins, (7) synthesis of proteins involved in the anti-oxidative stress response and (8) synthesis of the stress hormone ethylene. Since these defence mechanisms are already reviewed (di Toppi and Gabbrielli, 1999; DalCorso *et al.*, 2008; Seth *et al.*, 2012), only the most essential steps will be discussed in the next paragraph.

In general, the plants' mechanisms for detoxification of heavy metals appear primarily to be involved in avoiding the build up of toxic concentrations at sensitive sites rather than developing proteins that resist the heavy metal effects. At root level, Cd molecules can be prevented from entering the cell simply by immobilisation on the cell wall (on pectic sites and histidyl groups) and extracellular carbohydrates (Verkleij and Schat, 1990; Wagner, 1993). However, since the adsorption capacity of the cell wall is limited and since metal-specific tolerance is hard to explain by this mechanism, its role in metal tolerance is controversial (Hall, 2002). Next, root exudates such as malate and citrate can complex Cd to the soil matrix and thereby prevent its uptake by the plant (Delhaize and Ryan, 1995). Exclusion of Cd molecules at the level of the

cell membrane, could be effective, but is however not yet understood in plants. Cd is known to influence the lipid composition of membranes which may have a direct effect on the membrane permeability (Hall, 2002). Upon high toxicity, disruption of the plasma membrane can occur (Vangronsveld and Clijsters, 1994; Smeets *et al.*, 2005; Cuypers *et al.*, 2009; Cuypers *et al.* 2011), resulting in cellular leakage. When the concentration of the toxic element rises above the capacities of these barriers, active defence mechanisms are addressed to produce chelating compounds (glutathione, phytochelatins, metallothioneins, organic acids, …) that are involved in the detoxification and compartmentalization of Cd into specific cellular components. Besides the limited capacity of exclusion mechanisms, the presence of metal ion transporters make Cd entrance into the cell possible. Several metal ion transporters (i.e. ZIP, NRAMP, HMA, CAX, CDF and ABC transporters) are believed to be involved in the response to Cd, although hard evidence is often still missing (DalCorso *et al.*, 2008; Villiers *et al.*, 2011). Influx transporters are generally considered to show low selectivity, while intracellular transporters are highly selective in exporting metal ions from the cytosol to the vacuoles or outside the cell. The above mentioned transporters are involved in Cd uptake by the roots, Cd transport within the plant, xylem unloading processes or in exporting Cd out of the cytosol into the vacuole or the apoplast (DalCorso *et al.*, 2008; 2010). In contrast to the extensively studied vacuolar transporters, the soluble proteins in this organelle have drawn less attention. Considering that mechanisms following ion uptake in the vacuole and leading to its stabilization could be crucial for long-term storage and sequestration (Villiers *et al.*, 2011), this is an interesting field yet to be explored. Upon Cd entry in the cytosol, the production of phytochelatins is promptly activated (di Toppi and Gabbrielli, 1999; Horemans *et al.*, 2007; Semane *et al.*, 2007; DalCorso *et al.*, 2008). For long time, activation of phytochelatin synthesis has been considered as a result of direct interaction of the metal with the enzyme system. However, an alternative model is postulated, presuming an indirect activation by metal binding on the enzyme’s substrate (Vatamaniuk *et al.*, 2000). Since there are observations conflicting with this second model (Cobbett and Goldsbrough, 2002), more research needs to be conducted to elucidate the activation mechanism. Although misleading by their name, phytochelatins are found in plants as well as in animals. They are 1.5 – 4

kDa complexes with a general structure of (ϒ-Glu-Cys)n-X with n being a variable number from 2 till 11 and X generally being Gly, but variants containing β-Ala, Ser, Glu or Gln have been reported (Cobbett and Goldsbrough, 2002; Dalcorso, 2008, Seth *et al.*, 2012). In vitro studies with crude extracts and partially purified protein preparations showed that phytochelatin synthases are strictly dependent on the presence of metal or metalloid ions in the assay buffer, with Cd ions as the most potent activators. Phytochelatins chelate the free Cd molecules, preventing them from circulating in the cytosol (Grill *et al.*, 1985). Binding to these phytochelatins will finally lead to Cd compartmentalisation in the vacuole, generally accepted as the main site of metal sequestration in roots (Clemens, 2006b). To enter the vacuole, Cd-phytochelatin complexes will bind acid-labile sulphide (S^{2-}), probably at the tonoplast level. By the incorporation of sulphide into the Cd-phytochelatin complex, a HMW (high molecular weight) complex is formed. The incorporation of the sulphide increases the complex stability, the affinity to Cd and the amount of Cd per molecule (Cobbett and Goldsbrough, 2002). In yeast, it has been demonstrated that the transport of HMW complexes across the tonoplast is mediated by ATP-binding cassette (ABC) transporters (Cobbett and Goldsbrough, 2002). However, up to date no plant vacuolar ABC transporter has been identified transporting Cd-glutathione or Cd-phytochelatin complexes (Martinoia *et al.*, 2007). In the vacuole, due to the acid pH, the HMW complex will dissociate and Cd will be complexed by organic acids or amino acids present in the vacuole. The apo-phytochelatins will either be degraded inside the vacuole or return to the cytosol to fulfil their shuttle role. Phytochelatins play a decisive role in Cd detoxification at any concentration and exposure time, however an excessive amount of phytochelatins does not confer, *per se*, any hyper-tolerance (DalCorso *et al.*, 2008). A positive correlation between (1) the level of Cd exposure and exposure time and (2) the number of ϒ-Glu–Cys repeat units in phytochelatins was reported (Grill *et al.*, 1987; Vögeli-Lange and Wagner, 1996). Nevertheless, other factors might play a more important role than the phytochelatin level: (1) the efficiency of Cd detoxification seems to depend on the capacity of phytochelatins to link S^{2-} groups rapidly (di Toppi and Gabbrielli, 1999), (2) a FAD/NAD-linked disulphide reductase (perhaps a phytochelatin reductase) seems to be essential to guarantee sufficient reducing power to prevent Cd-induced phytochelatins to

become oxidized and to become inefficient to bind Cd (di Toppi and Gabrielli, 1999) and (3) the HMT2 protein is required to accumulate phytochelatins in the vacuole (Vande Weghe and Ow, 1997). Studies on the quantitative contribution of the phytochelatin pathway to Cd accumulation strongly argue for binding of a large portion of Cd by phytochelatin complexes. However, the binding capacity seems to be dose-related; at environmentally realistic Cd exposure (0.1 µM), only 50% of the total Cd in roots was bound, while at higher Cd exposure, the fraction on total Cd bound rose close to 90%. In leaves, only 27% of the total Cd was complexed by phytochelatins, indicating a slighter contribution of the phytochelatin pathway (Rauser, 2003). On the other hand, phytochelatins may play other important roles in the cell, including essential heavy metal homeostasis, sulphur and iron metabolism or anti-oxidant activity (Rauser, 1995; Dietz *et al.*, 1999; Cobbett, 2000). As mentioned above, another function reported for phytochelatins, is their role in Cd transport from root to shoot (Gong *et al.*, 2003). To keep Cd accumulation low in the root, a phytochelatin dependent 'overflow protection mechanism' acts to transport extra Cd to the shoot (Gong *et al.*, 2003). Although the phytochelatin pathway seems to be a promising response to Cd toxicity, the metabolic fate of phytochelatin-Cd complexes is poorly understood. On top, there are indications that these complexes are only transiently formed (Clemens, 2006b). Subsequently, the binding partners for long-term Cd accumulation are yet to be determined. Next to the phytochelatin-dependent sequestration into the vacuole, the Cd^{2+}/H^{+} antiport system across the tonoplast is also involved in Cd compartmentalisation (Clemens, 2006b). In mammalian cells, metallothioneins, low molecular weight cysteine-rich peptides, are known to bind Cd efficiently on cysteine residues via mercaptide bounds. In contrast to phytochelatins, metallothioneins (8 – 14 kDa complexes similar to those of meta 40-thioneine) are mRNA translation products. Although there is some evidence that supports their participation in Cu homeostasis (Cobbett and Goldsbourgh, 2002) and in metal ion transport (Garcia-Hernandez *et al.*, 1998; DalCorso *et al.*, 2010), there is no direct molecular evidence indicating a binding *in planta* of other metals than possibly Cu (Clemens, 2006b). Therefore, their role in Cd detoxification seems to be of minor importance. However, since a function as anti-oxidants and a role in plasma membrane repair is postulated, they could be indirectly involved in metal

tolerance (Salt *et al.*, 1998; Dietz *et al.*, 1999). As a secondary response strategy to Cd stress, transcriptional regulation of metal-responsive genes is described. Transcription factors putatively responsive to heavy metal stress have been identified (Fusco *et al.*, 2005). The generally accepted hypothesis of a highly complex plant response to Cd could be confirmed by the observation that the Cd-responsive transcription factors belong to different groups (DalCorso *et al.*, 2008). DNA of Cd stressed cells produce specific mRNA transcripts which regulate the synthesis of stress proteins. Stressed by Cd, plant cells start the synthesis of heat shock proteins (HSPs), more generally referred to as stress proteins since they are known to be induced by a variety of stresses (Villiers *et al.*, 2011). HSPs are involved in protein (re)folding, proteins stabilization under stress conditions and/or denaturation of misfolded proteins (di Toppi and Gabbrielli, 1999; Hall, 2002; Villiers *et al.*, 2011). Being a non redox-active metal, Cd can only evoke oxidative stress via indirect mechanisms; e.g. through disruption of the electron transport chains, the induction of lipid peroxidation and the induced changes in the anti-oxidant defence mechanism (Cuypers *et al.*, 2011). Hydrogen peroxide (H_2O_2) is believed to be a key molecule able to trigger signal transduction events after plant metal exposure (Smeets *et al.*, 2008a, 2009; Cuypers *et al.*, 2011). As a response, the anti-oxidative defence machinery needs to be enhanced in order to prevent cell death due to lipid peroxidation, protein oxidation, enzyme inhibition and damage to nucleic acid generated by oxidative stress. This defence machinery comprises non-enzymatic (glutathione, GSH; ascorbic acid, AsA; α-tocopherol and carotenoids) and enzymatic (superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; glutathione reductase, GR; monodehydroascorbate reductase, MDHAR; dehydroascorbate reductase, DHAR; peroxidases and glutathione-S-transferase, GST) anti-oxidants (Mittler *et al.*, 2004; Gill and Tuteja, 2011, Seth *et al.*, 2012). Finally, it is described that Cd stimulates ethylene production by enhancing the *in vivo* activity of ACC synthase (di Toppi and Gabrielli, 1999). Together with cysteine peptidases, serine peptidases, calcium and oxidative stress, ethylene is a main player in the Cd-induced cell death signalling (Iakimova *et al.*, 2008). Recently it was suggested that ethylene plays an important role in S-induced alleviation of Cd stress on photosynthesis (Masood *et al.*, 2012). However, due to the scarcity of data available, it is impossible to

understand the relationship between Cd stress and ethylene biosynthesis. To conclude, it should be mentioned that although Cd^{2+}-specific response genes are identified, most of the other responses identified to date have been classified as 'general stress responses'. These are known to be induced by various biotic and abiotic stresses in a large number of independent studies (Clemens, 2006b). Revealing the signalling events that lead to the activation or down-regulation of genes is crucial, since this is generally characterized as a 'black box' at this stage.

Chapter 1.3

Approaches to cope with cadmium contaminated sites

1.3.1 Which approach to choose?

Due to its relatively high mobility in soils, Cd accumulates significantly in plants grown on Cd contaminated soils, posing a serious threat to human and animal health. As mentioned above, it's easy entrance into the food chain leads to drafting threshold values for agricultural soils and crops. Due to the toxic effects of Cd, care must be taken into account when growing crops on Cd contaminated land. Moreover, highly contaminated land needs to be remediated before agricultural activities can be resumed. The different approaches that can be used on Cd contaminated soils can be categorized into two main groups: those limiting the uptake of Cd into the plants and those cleaning up the contaminated soil.

Appropriate management practices for metal contaminated sites, must aim at minimizing the risk of contaminant dispersal into the environment. Additives (e.g. lime, zeolite, phosphates, apatites, red mud, chemical chelators, ...) can be applied to avoid contaminant uptake by plants (Mench *et al.*, 1994; Ruttens *et al.*, 2006; Ruttens *et al.*, 2010). They are able to reduce the metal availability to the plant and subsequently reduce their toxic effects, resulting in a revegetation of contaminated land. By establishing a vegetation cover, further horizontal and vertical spreading of the contaminant is strongly reduced (Vangronsveld *et al.*, 1995, 1996, 2009). Moreover, in terms of phytoextraction, additives can be used to make soil metals more available for plant uptake (Meers *et al.*, 2008). Since chemical additives are very expensive, a recent shift occurred towards biofertilizers. Additionally, biodegradable amendments receive greater attention since some amendments typically show long term persistence. Although the use of additives can be beneficial, there is a risk of (1) uncontrolled dissolution and leaching, which can cause ground water pollution, (2) evoking toxic effects on microorganisms, (3) affecting soil solution pH and microstructure and of (4) enhancing mobilization and 'plant availability' of the contaminants (Nachtegaal *et al.*, 2005; Meers *et al.*, 2008; Vangronsveld *et al.*, 2009; Meers *et al.*, 2010).

Controlled Deficit Irrigation (CDI), is proposed as a technique to avoid pollutant leaching (Fine *et al.*, 2012). Besides the risks, the application of chelating agents is often promising on a small laboratory scale, but is much less efficient in the field (Glick, 2010). In their review on chelant-assisted phytoextraction, Evangelou *et al.* (2007) stated that possibly a turning point is reached in which chelant-assisted phytoextraction should be abandoned in favour of alternative options. Nonetheless, scientists propose this technique as a temporary measure while attending a more definitive remediation (Vangronsveld *et al.*, 2009; Simmler *et al.*, 2012). Lessons from the past should be considered using amendments. New Zealand, for example, copes with increasing Cd pollution caused by applying super phosphate fertilizers on the land (in past and present). To avoid introducing new potential risks, high care should be taken when using new amendments since their potential risks or negative characteristics often remain unexplored and underestimated. On top, these risks often only appear after several years of application.

As a more moderate strategy to limit Cd uptake and/or translocation in plants, plant breeding programs have utilized natural genetic variation within crop species to select and breed crop plants which accumulate low Cd in the grain or other edible plant parts. Although promising results have been obtained, there are constraints to its use since it is very time-consuming to develop suitable cultivars (5-10 years on average) (Grant *et al.*, 2008). Another strategy to avoid Cd contamination in food is the use of plant nutrients to alleviate Cd toxicity. Several plant nutrients have many (in)direct effects on Cd availability and toxicity (Sarwar *et al.*, 2010). It has long been known that an excess of essential cations such as Zn^{2+} and Ca^{2+} has a protective effect against Cd^{2+} toxicity (Antonovics *et al.*, 1971). Management of plant nutrients can be useful in (1) reducing Cd availability in soil, limiting root absorption, decreasing root-to-shoot translocation and (2) the development of tolerance against Cd toxicity and the reduction of Cd accumulation in edible plant parts. For a review on the relationships between plant nutrients and Cd, we refer to Sarwar *et al.* (2010). Focussing on sulphur nutrition, a review discussing the regulatory mechanism of S uptake and assimilation, GSH and PC synthesis in order to tolerate Cd stress, is provided by Gill and Tuteja (2011).

Clean up strategies are often chosen on Cd contaminated soils. Among them, a distinction can be made between “classical” and “new age” remediation techniques. The classical techniques (e.g. soil washing, excavation and dumping, the use of organic or inorganic amendments) are generally more invasive; they are more harmful for the environment, expensive, time consuming (Glick, 2010) and on top, they are - from an economic point of view - not applicable on large scale, diffuse contaminations (Meers *et al.*, 2008; Weyens *et al.*, 2009d). On the contrary, the “new age” techniques (e.g. phytoremediation, bacteria based remediation and a combination of the former) try to avoid negative effects on the total environment. These recent techniques have come a long way in a short time (Glick, 2010) and will be the focus of the next paragraph.

1.3.2 Phytoremediation

1.3.2.1 General information

Phytoremediation s.l. comprises the use of plants that exclude metals from edible plant parts (phytoexclusion) or to remove (phytoextraction), convert and/or degrade (phytotransformation) or stabilize (phytostabilization) contaminants in water or soils. During the last decade, these techniques gained increasing attention since they are considered to be cost-effective, environmentally friendly alternatives to the classical remediation techniques (Vangronsveld and Cunningham, 1998; Lasat, 2002, Vassilev *et al.*, 2004; Glick, 2010).

In contrast to organic contaminants, toxic metals can’t be degraded to non-hazardous compounds. Therefore, focussing on toxic metal contamination sites in the Northeastern part of Belgium, phytoextraction is considered to be a promising green remediation technique (Chaney, 1983). Using phytoextraction, pollutants are removed from contaminated soils by absorption into the plant and translocation to harvestable plant parts. The preconceived goal is to reduce the metal concentration to an acceptable value within a reasonable timeframe (do Nascimento *et al.*, 2006). Despite the growing interest in this technique, some limitations need to be considered: (1) the ‘plant available’ fraction of the metal in the soil, (2) the physical availability of the metal to the plant roots, (3) the translocation to the above ground plant parts and (4) the tolerable metal concentration within the plant. On top, although extensive efforts have been

made to use phytoextraction for removal of toxic metals from the soil, no convincing field data have been produced to allow its broad practical application as a short-term alternative for classical remediation techniques (Meers *et al.*, 2010).

1.3.2.2 Phytoextraction of toxic metals

1.3.2.2.1 Hyperaccumulators versus high biomass producing plants

In general, two approaches are applied in phytoextraction experiments: (1) the use of hyperaccumulators; focussing on Cd, this is defined as plants that are able to accumulate at least 100 mg kg^{-1} Cd in natural environments (Brown *et al.*, 1994; Reeves and Baker, 1999) or (2) the use of high biomass producing plants with a rather low metal accumulation capacity and low translocation efficiency. Although hyperaccumulators are able to accumulate high concentrations of toxic metals and are characterised by a high translocation efficiency, their absolute produced biomass is rather low and their remediation is only effective in the top soil layer. Using fast growing, high biomass producing plants like poplar and willow, provides some additional advantages: (1) being phreatophytes their roots follow the soil water table, and by consequence deeper soil layers can be reached for remediation, (2) the produced biomass can be used as a bio-energy source, making the long-term remediation process economically more attractive, (3) despite the generally low translocation rate of non-hyperaccumulators (DalCorso *et al.*, 2008), the high transpiration rate, typical for poplars and willows, generates a high water 'flow through' resulting in a higher water (and toxic metal) uptake capacity and one can speculate on a higher translocation efficiency. Finding the right balance between high levels of metal uptake and high productivity remains a major challenge. On top, finding the appropriate remediation plant depends strongly on the characteristics of the contaminated soil (Evangelou *et al.*, 2012). In order to make remediation of metal-polluted soils effective, plants must be tolerant to one or more metals, highly competitive, fast growing and able to produce high aboveground biomass (Glick, 2010). Moreover, the opinion exists that metal phytoextraction will be more economically interesting if plants produce biomass with an economical value (Vangronsveld *et al.*, 2009). Nonetheless, hyperaccumulators posses sources of genes for the improvement of non-hyperaccumulator plants

(DalCorso *et al.*, 2008). By transferring the genetic potential responsible for hyperaccumulation from hyperaccumulator species to plants with appropriate traits for phytoremediation (e.g. poplar, willow and Indian mustard), the capacity for pollutant accumulation and tolerance could be enhanced in the latter (DalCorso *et al.*, 2008; Liang *et al.*, 2009). Although positive results in this topic have been reported (e.g. Zhu *et al.*, 1999; Bennet *et al.*, 2003; Eapen and D'Souza, 2005; Krämer *et al.*, 2007; Hanikenne *et al.*, 2008), more information is needed on the mechanism responsible for hyperaccumulation/-translocation. Up to date, the molecular genetic basis of Cd tolerance and hyperaccumulation has not yet been incontrovertibly identified. Further efforts need to be undertaken to unravel whether the genetic control of Cd tolerance/hyperaccumulation is polygenic or oligogenic/monogenic (di Toppi and Gabrielli, 1999). Since there is no evidence of the occurrence of one single mechanism that can account for tolerance/hyperaccumulation to a wide range of metals (Hall, 2002), breeding plants for broad phytoremediation purposes will involve a large number of genetic changes (Macnair *et al.*, 2000). On top, it is not to be forgotten that the control can be both genetic as well as environmental (Baker *et al.* 1990).

The debate whether hyperaccumulators or high biomass producing plants should be used for phytoextraction, is still ongoing. Vangronsveld *et al.* (2009) pointed out that the choice of the phytoextractor depends on the site characteristics, and reviewed the advantages and disadvantages of both options.

1.3.2.2.2 Phytoextraction efficiency determinants

In order to determine the efficiency of the phytoextraction process several parameters should be considered (Lebeau *et al.*, 2008): (1) plant biomass, (2) metal concentration in the plants, (3) plant available metals in the soil, (4) translocation factor (TF; defined as the ratio of metal concentration in the shoot (C_s) to that in the root (C_r): TF = C_s / C_r) and (5) bio-accumulation factor. All these parameters will result in a certain phytoextraction rate (PhR; defined as PhR = (C_p x M_p / C_{so} x M_{rz}) x 100% where C_p is the metal concentration in the aboveground plant parts, M_p is the mass of the aboveground plant parts, C_{so} is the metal concentration in the soil and M_{rz} is the mass of the soil volume rooted by the species under study). Concerning the plant biomass and the metal

concentration within the plant, a trade off should be made between hyperaccumulators and high biomass producing plants (see section 1.3.2.2.1). As stated above, the amount of metals extracted by the plant, the translocation factor, the bio-accumulation factor (BF) and the phytoextraction rate are limitations that need to be overcome in order to render phytoextraction the efficiency it needs to become a widely applied remediation technique in metal contaminated sites. The definition of the bio-accumulation factor is not that straightforward. It is generally defined as: $BF = C_p / C_{so}$ where C_{so} is the metal concentration in the soil. However, depending on the author's interpretation, C_p can be defined as metal concentration in all aboveground plant parts or be divided into metal concentration in different plant parts i.e. root, stem and leaves. Liang *et al.* (2009) stated that the BF is species dependent and might change with soil composition and contaminant concentrations; the BF values for the hyperaccumulators *T. caerulescens* and *A. halleri* decreased with increasing soil metal concentration. This further raises the question whether hyperaccumulators are suitable for phytoextraction purposes. To screen poplar and willow plants for phytoremediation capacities, Zacchini *et al.* (2009) calculated the tolerance index as well (TI; $TI = (DW_{Cd} / DW_{con}) \times 100$ where DW_{Cd} and DW_{con} represent the dry weight of plants grown on Cd and control conditions respectively) and found poplars to be moderately tolerant to Cd, compared to willows which were highly tolerant.
In order to boost these limiting parameters, the exploitation of the interaction between plants and their associated microorganisms received increasing attention in the last decade and is termed 'bacterial enhanced phytoextraction'.

1.3.3 Bacterial enhanced phytoextraction

1.3.3.1 Plant-associated bacteria

Up to date, all investigated plants have shown to live in close relationship with microorganisms (e.g. bacteria and fungi). These microorganisms can support nutrient uptake, increase resistance against pathogens and enhance plant growth (Mathesius, 2009). Focussing on plant-associated bacteria, two classes can be distinguished: rhizosphere bacteria and endophytic bacteria. Rhizosphere bacteria live in close relationship with the plant at the rhizosphere level. They thrive on root exudates and in return help their host plant to cope with various

stresses, including metal contamination. Here, it should be mentioned that at rhizosphere level, mycorrhiza and more precisely ectomycorrhiza, can also contribute to ameliorating the effects of metal toxicity in host plants (Hall, 2002; Krznaric *et al.*, 2009; Krznaric *et al.*, 2010; reviewed by Miransari, 2010). Endophytic bacteria colonize the internal tissues of their host plant and can form a range of different relationship states including symbiotic, mutualistic, commensalistic and trophobiotic (Ryan *et al.*, 2008). Bacteria can switch between different relationship states and it has been postulated that many symbiotic or pathogenic bacterial taxa live as commensals on the plant before they undergo a closer association (Knief *et al.*, 2011). The switch seems to be based on mechanisms that coordinate the expression of genes whose products ultimately determine the nature of the association (Knief *et al.*, 2011). On top, it should be mentioned that this distinction (rhizosphere vs endophytic bacteria) is not always as straightforward since a significant part of endophytic bacteria originate from the rhizosphere (Ryan *et al.*, 2008). Endophytes are considered to enter the roots through cracks at the point of lateral root formation and to subsequently colonize the root intercellular space, aerenchym and cortical tissues. Only few bacteria enter the stele to colonize other plant parts. As a result, the endophytic concentration is highest in the roots, followed by the stem and the leaves (Compant *et al.*, 2010; Weyens *et al.*, 2011a). In addition, plant endophytic bacteria can be transferred to subsequent generations via seeds (Mastretta *et al.*, 2009; Remans *et al.*, 2012), by colonization of meristems (Pirttilä *et al.*, 2000), by transfer through gametes (Madmony *et al.*, 2005) or through direct vascular connections from the maternal plant (Block *et al.*, 1998). Using plant-associated bacteria, investigators hope to circumvent the limitations specific to phytoextraction that are listed above. The use of soil bacteria to facilitate phytoremediation is reviewed by Glick (2010). By producing organic acids or siderophores, plant-associated bacteria (rhizosphere or endophytic) can influence the availability of the metal(s) in the soil in response to the plant's need (Shenker *et al.*, 2001). Furthermore, it is described that endophytic bacteria are able to play a role in the translocation of metals to the aboveground plant parts (Lodewyckx *et al.*, 2002a,b; Mastretta *et al.*, 2006). On top, many plant-associated bacteria have been reported to promote plant growth directly or indirectly (Lodewyckx *et al.*, 2002a; Lucy *et al.*, 2004; Lebeau *et al.*, 2008;

Weyens *et al.*, 2009a, Remans *et al.*, 2012). Direct stimulation of plant growth occurs by the production of phytohormones (e.g. cytokinines, auxines), by the fixation of nitrogen from the atmosphere, by the production of siderophores which make essential nutrients available to plants, by producing enzymes that influence plant growth and development (Glick, 2003) or by inducing ACC deaminase activity leading to a decreased ethylene production (Glick, 2005; Arshad *et al.*, 2007). Indirect stimulation is accomplished by competition with pathogens for nutrients and space (Glick, 2003; Selosse *et al.*, 2004), by producing antibiotics (Rosenbleuth and Martinez-Romero, 2006), by producing hydrolytic enzymes that attack pathogen cell walls, by deactivating toxic compound produced by pathogens or by inducing the plant's defence mechanisms (Selosse *et al.*, 2004; Weyens *et al.*, 2009a). By enhancing plant growth, a larger root system is established and subsequently the volume of soil that can be reached is enhanced. In this context, Arshad *et al.* (2007) stated that 'extensive root growth is a prerequisite to maximizing the effectiveness of phytoremediation processes'. On top, generating more biomass results in a higher absolute amount of metals that can be stored before phytotoxic effects occur, referred to as the 'dilution effect' and in a higher production of potential bio-energy.

1.3.3.2 Engineered plant-associated bacteria

Another approach, next to exploiting the characteristics inherent to a specific plant-associated bacterium, is to equip bacteria with extra traits in order to enhance the clean-up of a specific contaminant (Barac *et al.*, 2004; Taghavi *et al.*, 2005; Andria *et al.*, 2009; Weyens *et al.*, 2009b; 2010a; 2010b; 2011b). Moreover, by combining specific 'contamination traits' with growth-promoting traits, remediation efficiency might be enhanced even more.

To cope with an excess of toxic metals, different mechanisms have been reported in bacteria: (1) enzymatic detoxification, (2) intracellular sequestration, (3) active efflux transport, (4) extracellular sequestration, (5) reduction in metal sensitivity of cellular targets and (6) metal exclusion (Bruins, 2000). By binding (precipitating) metal ions onto their cell wall or by intra- and extracellular sequestration, the 'plant availability' of the toxic metal decreases in the bacterial environment. This indicates that the bacterial mechanisms to cope with metal

stress could have a beneficial effect on phytoextraction efficiency. That is, if endophytic bacteria possess mechanisms to lower the 'plant availability' of Cd in the plant, the plant is able to take up more Cd from the contaminated soils before toxic effects will appear. Focussing on remediation of Cd contaminated soils, the CZC / CZR efflux mechanism is of special interest since it allows Cd ions to be precipitated onto the bacterial cell wall (Figure 1.3.1). The *czc* operon comprises 8 genes; 3 genes coding for structural proteins and 5 genes involved in regulation. The structural genes *czcC, czcB* and *czcA* code for proteins that together form a three component export pathway (Nies *et al.*, 1989). CzcC is required to complete the efflux of ions into the extracellular medium. CzcB is a transmembrane protein connecting the inner and outer membrane and thereby preventing leakage of ions into the intermembrane space. On top, it might also provide specificity for heavy metals (Nies *et al.*, 1989). CzcA, the centre part of the heavy metal efflux pump, is known as a chemiosmotic cation/H^+ antiporter (Taghavi *et al.*, 1997). Since the *czc* operon is inducible by zinc, cobalt and Cd, loci that regulate the transcription of the *czc* structural genes and that respond to these metals, must be present (Taghavi *et al.*, 1997). Usually control of heavy metal resistance determinants by their substrates is a simple process. However, compared to other metal resistance determinants, regulation of the cobalt/zinc/cadmium resistance in *Ralstonia metallidurans* is a complex process (Groβe *et al.*, 2004). For one, the regulation of the czc operon is influenced by cytoplasmic as well as periplasmic metal cation concentrations. Two regions have been identified that might be involved in the heavy metal-dependent regulation of the *czc* operon. The upstream region comprises 2 regulatory genes, i.e. *czcN* and *czcI*. Transcription of both genes is induced by 300 µM zinc and by 300 µM cobalt. In contrast, transcription was not elevated in the presence of 300 µM Cd (Groβe *et al.*, 1999). It is known that *CzcN* contains a CzcR binding site (Groβe *et al.*, 2004), but as for *czcI*, its precise function is not yet understood (Groβe *et al.*, 1999; von Royzycki and Nies, 2009). Downstream of the structural genes *czcCBA*, a second regulatory locus i.e. *czcDRSE,* is found (Nies, 1992, Nies *et al.*, 1989, van der Lelie *et al.*, 1997). The precise function of CzcD is not yet known, but it is believed to be a transmembrane spanning protein, more specific a cation diffusion facilitator (CDF). Functioning as a metal sensor, CzcD may play a role in expression of the CzcCBA pump (Legatzki *et al.*, 2003;

Groβe *et al.*, 2004). The CzcRS is considered to act as a two component regulatory system in which CzcS acts as the sensor histidine kinase and CzcR as the response activator (van der Lelie *et al.*, 1997). CzcR is known to bind to the *czcNp* promoter region, but not to any other *czc* promoter (Groβe *et al.*, 1999). CzcE is a periplasmic, metal-binding protein, which is believed to function as an indirect repressor of the *czcNp* promoter. It is recently speculated that CzcE may inhibit phosphorylation of CzcS at low periplasmic metal-ion concentrations, resulting in a CzcE -> CzcS -> CzcR -> *czcNp* transduction chain (Groβe *et al.*, 2004). As a consequence of the cation/H^+ antiporter, high metal concentrations are present at the cell membrane and pH increases in this area. In this pH gradient, carbon dioxide, produced by the cellular carbon metabolism, is transformed into carbonates and bicarbonates. These (bi)carbonates precipitate with the metal cations onto the bacterial cell wall, preventing metal ions to re-enter the bacterial cell (Diels *et al.*, 1993). This bioprecipitation process can be of great interest for phytoextraction purposes: endophytic bacteria equipped with the *czc* operon might be able to lower the amount of 'plant available' Cd within the plant by binding it onto their cell wall.

In *Pseudomonas aeruginosa* CMG103 a metal resistant mechanism, showing great similarity with the metal resistance mechanism encoded by the *czc* operon, was identified. This *czr* operon, involved in zinc and Cd resistance, comprises 5 genes: *czrSRCBA*. The gene products of *czrCBA* resemble the chemiosmotic cation-proton antiport efflux system of the *czcCBA* gene cluster. On top, the predicted CzrS and CzrR proteins show a significant similarity to the sensor and regulatory proteins of the two component regulatory systems CzcS/CzcR respectively. A major difference between the *czr* system and its *czc* counterpart is the location of the regulatory genes and their direction of transcription (Hassan *et al.*, 1999). Aiming to introduce the above described three component metal resistance mechanism in plant growth-promoting bacteria, the *czr* system has an advantage over the *czc* system since it is easier expressible outside its host (van der Lelie D, personal comment).

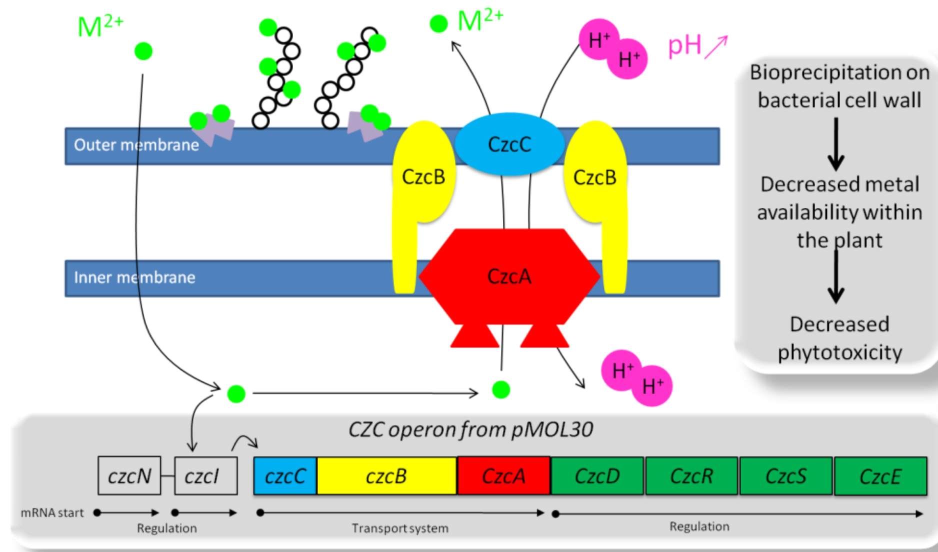


Figure 1.3.1: czc metal resistance mechanism. A three component metal export mechanism, consisting of CzcC, CzcB and CzcA proteins, is responsible for metal (M^{2+}) export. Due to the metal/proton antiport system, pH decreases, resulting in a metal precipitation on the bacterial cell wall. Two zones of regulatory genes, one upstream and one downstream, are involved in this bacterial defence system.

Although engineered plant-associated bacteria gain increasing attention in the field of phytoremediation, scientists remain sceptic about their application in the field: Singer *et al.* (2005) stated 'notwithstanding the phenomenally large and ever increasing resource of pollutant-degrading microbial isolates in laboratories around the globe, inoculum survival remains the Achilles' heel for bio-augmentation of contaminated land'. It is irrefutable that inoculated strains need to compete with the natural bacterial population. In the field of bio-augmentation, priming i.e. predisposing an isolate or population of microorganisms to future conditions in which they are designed to perform a role, is however believed to be a promising technique to give the inoculum a higher survival chance (Weyens *et al.*, 2009b). However, it is noteworthy to mention that it might even not be necessary for the inoculated strain to survive in order to obtain satisfactory remediation results. Due to horizontal gene transfer the natural endophyte population could be equipped with the capacity to

degrade or stabilize a pollutant without requiring long-term establishment of the inoculated strain (Ryan *et al.*, 2008). On top, the endophytic niche is considered a hot spot for horizontal gene transfer (Taghavi *et al.*, 2005; Weyens *et al.*, 2009b; Weyens *et al.*, 2009d).

Section II:

OBJECTIVES

2.1 Objectives

The work presented in this thesis is devided into two parts:

The first part focuses on engineering Cd-resistant plant growth-promoting endophytes to enhance phytoextraction

Comparing proteomes of non-inoculated poplar plants to those of poplar plants inoculated with engineered Cd-resistant endophytes, in control and Cd-exposed conditions, will contribute to unravel the molecular mechanisms underlying bio-augmented assisted phytoextraction. Aiming to conduct this research in the future, plant growth-promoting bacteria (PGPB) *P. putida* W619 and *Enterobacter* sp. 638 were equipped with the czr metal resistance mechanism via horizontal gene transfer (chapter 3.1). Subsequently, engineered stains were investigated for their (1) phenotypical characteristics, (2) capacity to extract Cd from liquid medium and (3) capacity to extract Cd from contaminated soil. In a final experiment, their effects on poplar cuttings exposed to Cd was examined in a greenhouse experiment.

The second part focuses on proteomic research to reveal a basic understanding of the differences induced by plant cultivation systems.

Prior to the proteomic analysis, an optimization of the proteomics workflow was conducted for *P. deltoides x* (*trichocarpa x deltoides*) (chapter 4.1). Next, literature was studied to get more information concerning the effects of Cd on the plants' proteome. From this search, it became obvious that a comparison between experiments focussing on Cd-induced changes in plant proteomes is not that straightforward. Therefore a meta-analysis focussing on plants' response to Cd in different cultivation conditions was conducted (chapter 4.2). Herein the encountered obstacles were discussed and a comparison between hydroponic and soil cultivation systems was made. However, since a large diversity among experimental conditions was present, the observed differences could not solely be attributed to the cultivation systems. Data obtained from the meta-analysis, together with the suggestions about differences caused by cultivation systems (Durand *et al.*, 2010) and the fact that, to our knowledge, no study has yet been conducted comparing the effects of hydroponic and soil cultivation systems, led

us to perform a profound study to address the question whether or not hydroponically obtained data can be used to make hypotheses for responses of field grown plants. It indeed is very important to have a basic understanding of the differences induced by growing plants in hydroponic cultivation systems compared to those grown in soils. To gain more insights in this matter, a study was conducted to map the effect of Cd on the proteome of poplar cuttings grown on hydroponic (chapter 4.3) and soil cultivation systems (Chapter 4.4) after short and long term exposure. Finally, these results were compared to reveal the effects of cultivation systems and to verify if results obtained on hydroponic cultivation systems can be used to postulate hypotheses for field studies (chapter 4.5).

Section III:

BACTERIAL ENHANCED PHYTOEXTRACTION

Chapter 3.1

Equipping plant growth-promoting bacteria (PGPBs) with a metal resistance system: effects on metal uptake and translocation in poplar

3.1.1. Introduction

Different plant species (i.e. tobacco, rapeseed, maize, sunflower, willow and poplar) have been tested on the metal contaminated soils in the Campine region in the NE of Belgium to monitor their metal phytoextraction capacities. Mostly, high biomass producing and metal accumulating plants were proposed to serve as phytoextraction crops (Di Baccio *et al.*, 2003; Klang-Westin and Erikson, 2003; Laureysens *et al.*, 2004; 2005; Giachetti and Sebastiani, 2006; Pulford and Dickinson, 2006). Unfortunately, up to date no real "species of choice" could be pointed out and the idea of using different species complementary gains interest. Focussing on the contaminated soils in the Campine region, *Populus deltoides x (trichocarpa x deltoides)* cv. Grimminge was chosen for its rust-resistance and its growth capacities on acid, poor sandy soils, which are characteristic for this area. Moreover, poplar trees are generally known as high biomass producing plants with a high transpiration rate and a deep rooting system. In a recent study on a former maize field in Lommel (Belgium), it was indicated that 'in order to make phytoextraction, based on short rotation coppice plantations, realistic for application it would be necessary to obtain higher biomass production and/or higher plant metal concentrations' (Witters *et al.*, 2009).

To enhance the plant biomass production, plant growth-promoting bacteria (PGPB) can be considered. By stimulating plant growth, a more branched and extended rooting system is formed which (1) enhances the physical availability of the metal and (2) allows cleanup of a larger soil surface area and deeper soil layers. Moreover, a higher total biomass production (3) leads to a higher metal removal in absolute terms and (4) makes the economic aspects of phytoextraction more attractive. From a bacterial isolation study, *Pseudomonas putida* W619 became known as poplar-PGPB (derived from *Populus trichocarpa x*

deltoides cv. Hoogvorst; Taghavi *et al.*, 2005). Another well known poplar PGPB is the *Enterobacter* sp. 368, derived from *Populus trichocarpa x deltoides* cv. H11-11 (Taghavi *et al.*, 2010). They both posses several genetic traits indicating growth-promoting capacities (Table 3.1.1) and on top, they have been reported to be growth-promoting in greenhouse studies (Taghavi *et al.*, 2009; Weyens *et al.*, 2011a). Aiming to enhance phytoextraction of *Populus deltoides x (trichocarpa x deltoides)* cv. Grimminge in the Campine region, *Pseudomonas putida* W619 and *Enterobacter* sp. 368 were chosen as PGPB since they were extracted from poplar trees highly related to *Populus deltoides x (trichocarpa x deltoides)* cv. Grimminge.

Table 3.1.1: Genotypical traits of *Enterobacter* sp. 638 and *Pseudomonas putida* W619. Genotypical traits derived from Taghavi *et al.*, 2009.

	Enterobacter sp.638	*P. putida* W619
Autotrophy	-	-
Nitrogen fixation	-	-
D-mannitol	+	+
Lactose	+	-
Sucrose	+	+
Arbutin	-	+
Salicin	+	+
Pectin	-	-
Trehalose	+	+
D-Mannose	+	+
L-Arabinose	+	+
Xylose	+	+
Maltose	+	+
Cellobiose	+	+
Chitin	+/-	+
4-aminobutyrate	-	+
Phosphonoacetic acid	-	+
1-aminocyclopropane-1-carboxylic acid	-	-
Glucose	+	+
Gluconate	+	+

In order to increase the metal concentration within the plant, plant endophytes can be equipped with metal resistance mechanisms, as described in the introduction (see chapter 1.3, pp. 58 - 62). The CZR operon, found in *Pseudomonas aeruginosa*, is able to confer Cd resistance to bacteria. Moreover, due to the Cd/proton antiport, Cd is considered to be precipitated onto the bacterial cell wall (Diels *et al.*, 1993). The CZR operon highly resembles the CZC operon found in *Cupriavidus metallidurans* CH34 (Hassan *et al.*, 1999), but is less studied. As discussed in the introduction, PGPBs will be equipped with the CZR operon since it is easier expressible outside its host strain compared to the CZC operon.

As phytoextraction remains a long term strategy, the main objective is to maximally boost phytoextraction efficiency. This can be achieved on 3 levels: (1) cultivar / clone selection, (2) use of PGPB and (3) equipping the PGPBs with a metal resistance and moreover metal precipitating system. In this research, an attempt to improve phytoextraction will be done by inoculating *Populus deltoides x (trichocarpa x deltoides)* cv. Grimminge with *Pseudomonas putida* W619 or *Enterobacter* sp. 368; both equipped with the CZR operon. Therefore, prior to future inoculation experiments, *Pseudomonas putida* W619 and *Enterobacter* sp. 368 need to be equipped with the CZR operon and verified by characteristic tests and a greenhouse experiment in order to confirm if they maintained their growth-promoting capacities.

3.1.2. Materials and methods

3.1.2.1. Conjugation

To transfer the CZR operon to PGPBs, a triparental conjugation was conducted using 'H12' as a conjugative helper strain. Both *Pseudomonas putida* W619 and *Enterobacter* sp. 638 were conjugated with all donor strains (CM1600, CM1601, CM1602, CM1603 and CM1366) (Table 3.1.2). Acceptor and helper strains were cultivated in liquid rich medium (10 g l^{-1} peptone, 5 g l^{-1} yeast extract, 5 g l^{-1} NaCl, 1 g l^{-1} glucose D^+ and 0.345 g l^{-1} $CaCl_2*2H_2O$ in destilled water, final pH 7.0) overnight (30°C, 120 rpm). Donor strains were cultivated overnight in liquid rich medium with 0.4 mM Cd to select for Cd resistance. After centrifugation (30 min, 4000 rpm, RT), bacterial pellets were washed twice with 10 mM $MgSO_4$. Subsequently pellets were resuspended in 10 mM $MgSO_4$ till an

optical density of 0.5 was reached (A660). Triparental conjugation was performed on solid rich medium (1,5% agar) by mixing 80 µl donor, acceptor and helper strains. After overnight incubation at 30°C, bacteria were harvested in 1 ml 10 mM $MgSO_4$ from which a dilution series was made up till 10^{-4}. To select for transconjugant strains 100 µl of each dilution was spread onto solid (2% agar) 284 medium (6.06 g l^{-1} TRIS-HCl, 4.68 g l^{-1} NaCl, 1.49 g l^{-1} KCl, 1.07 g l^{-1} NH_4Cl, 0.43 g l^{-1} $NaSO_4$, 0.2 g l^{-1} $MgCl_2$*$6H_2O$, 0.03 g l^{-1} $CaCl_2$*$2H_2O$, 0.04 g l^{-1} Na_2HPO_4*$2H_2O$, 4.8 mg l^{-1} Fe(III)NH_4 citrate, 1 ml microelement solution and carbon mix in distilled water, final pH 7.0; microelement solution: 1.3 ml l^{-1} 25% HCl, 144 mg l^{-1} $ZnSO_4$*$7H_2O$, 100 mg l^{-1} MnCl*$4H_2O$, 62 mg l^{-1} H_3BO_3, 190 mg l^{-1} $CoCl_2$*$6H_2O$, 17 mg l^{-1} $CuCl_2$*$2H_2O$, 24 mg l^{-1} $NiCl_2$*$6H_2O$ and 36 mg l^{-1} $NaMoO_4$*$2H_2O$ in distilled water; carbon mix: 0.52 g l^{-1} glucose, 0.35 g l^{-1} lactate, 0.66 g l^{-1} gluconate, 0.54 g l^{-1} fructose and 0.81 g l^{-1} succinate) with 0.8 mM $CdSO_4$ and incubated for several days at 30°C till bacterial colonies were formed. For each conjugation, 10 colonies were selected, grown in liquid rich medium overnight (120 rpm, 30°C), centrifuged (6000 rpm, 10 min, RT), washed twice in 10 mM $MgSO_4$ and resuspended in a glycerol solution for storage at -80°C.

3.1.2.2. Phenotypical characteristics

3.1.2.2.1. Growth capacity on different media

Donor, helper and acceptor strains were tested for growth capacities on rich and 284 media with or without 0.4 or 0.8 mM $CdSO_4$, in order to determine the selective medium for transconjugant strains.

3.1.2.2.2. Cd resistance

After conjugation, transconjugant strains were tested for their Cd resistance. Each selected transconjugant strain (10 per conjugation) was spread onto solid 284 medium supplemented with 0.2, 0.4, 0.8, 1.2, 1.6, 2 and 4 mM $CdSO_4$ and incubated at 30°C for maximum 7 days.

3.1.2.2.3. Plasmid stability

Plasmid stability (pMOL888 or pMOL864) was checked in all transconjugant strains by growing the strains for 100 generations on non-selective liquid rich medium. The 100th bacterial generation was spread onto solid 284 medium

supplemented with $CdSO_4$ in a concentration to which the parental strain was resistant.

3.1.2.2.4. IAA production

Bacterial strains were grown in 5 ml liquid rich medium supplemented with L-tryptophan (0.5 g per liter) for 4 days in the dark (150 rpm, 30°C). After centrifugation (4000 g, 10 min, RT), 1 ml Salkowski reagent (49 ml 35% $HClO_4$ and 1 ml 0.5 M $FeCl_3$) was added to 0.5 ml supernatant and vortexed. After 20 minutes, strains producing IAA stained pink.

3.1.2.2.5. Organic acid production

Bacterial strains were tested for organic acid production according to the method of Cunningham and Kuiack (1992). In brief, after cultivation of bacterial strains in liquid rich medium, 20 µl of the bacterial suspension was added to 800 µl ST medium (20 g sucrose, 5 g tryptone and 10 ml trace element solution per liter distilled water; trace element solution: 20 mg l^{-1} $NaMoO_4$*$2H_2O$, 200 mg l^{-1} H_3BO_3, 20 mg l^{-1} $CuSO_4$*$5H_2O$, 100 mg l^{-1} $FeCl_3$, 20 mg l^{-1} $MnCl_2$*$4H_2O$ and 280 mg l^{-1} $ZnCl_2$ in distilled water). Subsequent to an incubation of 5 days (200 rpm, 30°C), 100 µl alizarine red (0.1% w/w) was added. After 15 minutes, strains producing organic acids stained yellow.

3.1.2.2.6. Siderophore production

To test bacterial strains for siderophore production, the method of Schwyn and Nielands (1987) was used. Bacterial strains were cultivated in liquid rich medium. Twenty microliter of the bacterial suspension was added to 800 µl liquid 284 medium supplemented with 0, 0.25 or 3 µM Fe(III) citrate and incubated for 5 days (200 rpm, 30°C). Four hours after the addition of 100 µl CAS reagent, strains producing siderophores stained orange.

Table 3.1.2: Bacterial strains used in triparental conjugation.

Donor strains		**Description**	**Chromosome**	**Plasmid**
CM1600		12,8 kb fragment of pMOL864 in pLAFR3 in *E. coli*		pMOL888
CM1601		12,8 kb fragment of pMOL864 in pLAFR3 in *E. coli*		pMOL888
CM1602	*Bacteria, proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae, Pseudomonas*	12,8 kb fragment of pMOL864 in pLAFR3 in *E. coli*		pMOL888
CM1603		12,8 kb fragment of pMOL864 in pLAFR3 in *E. coli*		pMOL888
CM1366		pMOL864 (pLAFR3::czr) in *E. coli*		pMOL864
Helper strain				
H12	*Bacteria, proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae, Pseudomonas*	Helper strain, containing F genes		Hfr
Acceptor strains				
P. putida W619	*Bacteria, proteobacteria, Gammaproteobacteria, Pseudomonadales, Pseudomonadaceae, Pseudomonas*	Growth-promoting bacteria, derived from *Populus trichocarpa x deltoides* cv. Hoogvorst	5.774.330 bp	-
Enterobacter sp. 638	*Bacteria, proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, Enterobacter*	Growth-promoting bacteria, derived from *Populus trichocarpa x detoides* cv. H11-11	4.518.712 bp	pENTE01

3.1.2.2.7. ACC deaminase production

Bacterial strains were tested for ACC deaminase production according to the method of Belimov *et al.* (2005). In brief, bacterial strains were cultivated in liquid BPF medium (10 g l^{-1} tryptone, 10 g l^{-1} casein hydrolysate, 12.5 g l^{-1} glycerol, 1.5 g l^{-1} KH_2PO_4, 1.5 g l^{-1} $MgSO_4$ in distilled water) for 48 h at 30°C. Bacterial suspensions were centrifuged (4000 rpm, 15 min, RT), washed twice in 0.1 M TRIS-HCl buffer (pH7.5) and resuspended in SM medium supplemented with 5 mM ACC and carbon mix (0.4 g l^{-1} KH_2PO_4, 2 g l^{-1} K_2HPO_4, 0.2 g l^{-1} $MgSO_4$*$7H_2O$, 0.1 g l^{-1} $CaCl_2$, 5 mg l^{-1} $FeSO_4$*$7H_2O$, 2 mg l^{-1} H_3BO_3, 5 mg l^{-1} $ZnSO_4$, 1 mg l^{-1} Na_2MoO_4, 3 mg l^{-1} $MnSO_4$, 1 mg l^{-1} $CoSO_4$, 1 mg l^{-1} $CuSO_4$, 1 mg l^{-1} $NiSO_4$ in distilled water, pH 6.6; carbon mix: 1 g l^{-1} glucose, 1 g l^{-1} sucrose, 1 g l^{-1} Na-acetate, 1 g l^{-1} Na-citrate, 1 g l^{-1} malic acid, 1 g l^{-1} mannitol). Following a second centrifugation (4000 rpm, 15 min, RT), bacterial pellets were resuspended in 0.1 M TRIS-HCl buffer (pH8.5) and cells were lysed with toluene. Cell suspensions were vortexed and 100 µl was added to 10 µl 0.5 M ACC and 100 µl 0.1 M TRIS-HCl (pH8.5). Mixtures without bacterial suspension or ACC served as negative controls. After an incubation period of 30 min (30°C), 1 ml 0.56 N HCl was added and samples were centrifuged (13500 rcf, 5 min, RT). Thereafter, 400 µl 0.56 N HCl and 150 µl 0.2% 2,4-dinitrophenylhydrazine (in 2 N HCl) was added to 500 µl supernatants. After a final incubation of 30 minutes (30°C), 1 ml 2 N NaOH was added and strains producing ACC deaminase stained brown.

3.1.2.3. Genetic characterisation

3.1.2.3.1. BOX PCR

After bacterial purification, total genomic DNA was extracted of each transconjugant strain using a DNeasy blood and tissue kit (Qiagen). Of the extracted DNA, 1 µl was used for BOX-PCR DNA fingerprinting (BOX1 primer: 5'-CTACGGCAAGGCGACGCTGACG-3') which was carried out as described in Barac *et al.* (2004) and Weyens *et al.* (2009c). PCR products were analysed by 1D gel electrophoresis (1.5% agarose) and visualized by Gel Red nucleic acid gel staining and UV illumination.

3.1.2.4. Cd extraction from liquid medium (Figure 3.1.1)

To test Cd extraction/precipitation abilities, bacterial strains were cultivated in liquid rich medium supplemented with 40 µM $CdSO_4$ for 3 days. Meanwhile, an aliquot of 250 ml liquid rich medium (control group; 'AQ1') and an aliquot of 250 ml liquid rich medium supplemented with 40 µM $CdSO_4$ (cadmium group; 'AQ2'), were prepared per strain and stored at 30°C to check for contamination. For both groups, reference samples were included. Reference samples consisted of the same medium as used in the group but no bacteria were added. After centrifugation of bacterial suspensions (4000 rpm, 20 min, RT), pellets were washed twice in 10 mM $MgSO_4$ and finally resuspended in 10 mM $MgSO_4$ (OD = 0.5; λ = 660). Subsequently, for each strain, reference and control samples the following handlings were performed on AQ1 and AQ2: 50 ml was taken to define pH and Cd concentration at time point zero. Cd concentration was measured by ICP-OES (Inductively Coupled Plasma – Optical Emission Spectrometry). Three aliquots of 50 ml (3 replicates) were taken from the remaining 200 ml and to these, 100 µl bacterial suspension (OD = 0.5; λ = 660) was added. To control and reference samples, 100 µl 10 mM $MgSO_4$ was added. After an incubation period of 2 days (120 rpm, 30°C), optical density was determined (λ = 660) of each replicate and replicates were subsequently centrifuged (4000 rpm, 30 min, RT). For each replicate, pH and Cd concentration at time point '4 days' were determined.

To compare differences in Cd removal between bacterial strains, the amount of Cd removed from the medium (µg) was expressed relative to the amount present on time point zero:

$$\frac{[Cd]T0 - [Cd]Tx}{[Cd]T0}$$

[Cd]T0 = concentration Cd on time point zero

[Cd]Tx = concentration Cd after 4 days of bacterial growth

Here of, reference values were subtracted to take into account possible Cd precipitation during the 4 days incubation. Further, the value was expressed relative to the bacterial unit present at harvest time. One bacterial unit corresponds to $1*10^{-8}$ bacteria. Control samples were not included since no Cd was detected after ICP-OES analysis.

Similar calculations were used to determine the differences in pH per bacterial unit. Reference value was subtracted to take the pH difference inherent to the medium into account. As for Cd concentration, the bacterial unit corresponds to $1*10^{-8}$ bacteria.

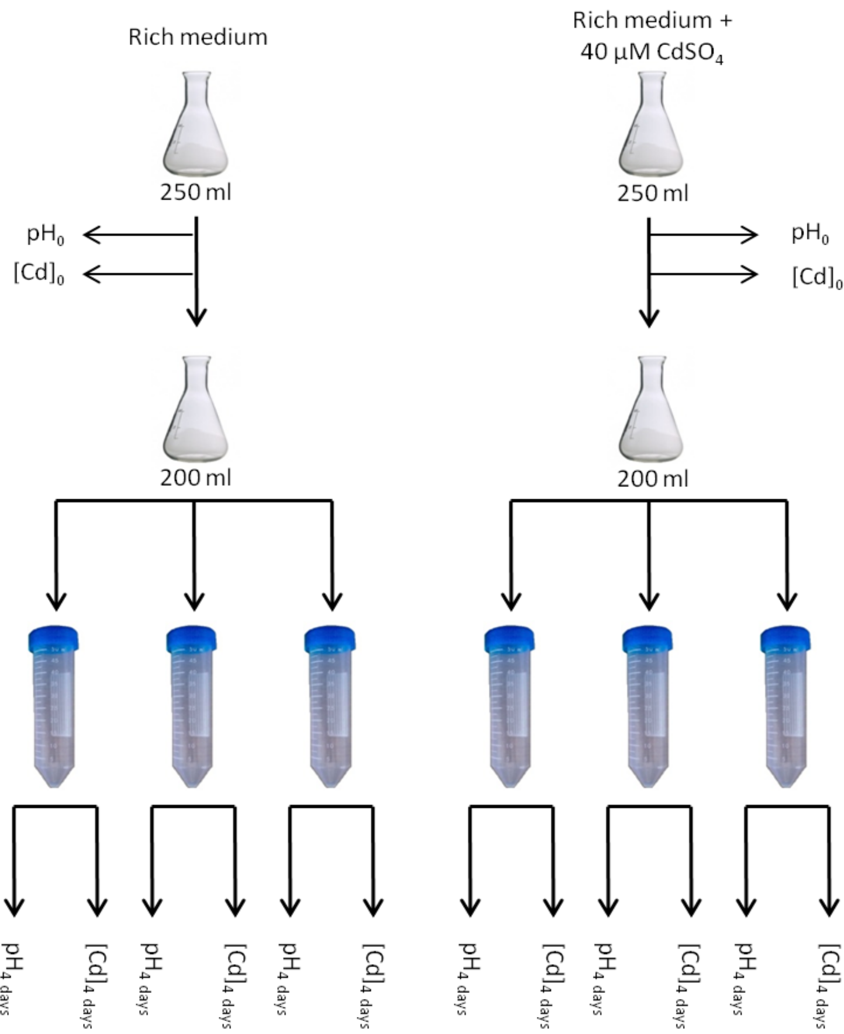


Figure 3.1.1: Cd extraction from liquid medium: schematic overview.

3.1.2.5. Cd extraction from contaminated soil

The protocol as described by Kuffner *et al.*, (2010) was used. In brief, bacterial strains were grown in liquid rich medium for 2 days. After centrifugation (4000 rpm, 20 min, RT), pellets were washed twice with 10 mM $MgSO_4$ and finally resuspended in 10 mM $MgSO_4$ (OD = 0.5; λ = 660). From this suspension, 100 µl was added to 10 ml 10% TSB (pH 7.2). Triplicates were made for each strain. Three replicates of 10 ml 10% TSB (pH 7.2) with 100 µl 10 mM $MgSO_4$ served as controls. Strains were cultivated till stationary growth phase was reached. Final optical density (λ = 660) was measured and samples were centrifuged (8000 g, 15 min, RT). Subsequently supernatants were filtered (0.2 µM), pH was measured and exactly 5 ml was transferred to 1 g sieved soil. Soil was obtained from a test field in Lommel, known to be contaminated with multiple metals, including Cd at a concentration of 5 mg kg^{-1} DW soil. Samples swirled for 2 h at 20 rpm and were then centrifuged (7000 rpm, 5 min, RT), filtered (0.5 µM) and analysed by ICP-OES.

In order to compare the results, Cd removal and pH difference were calculated as described in section 3.1.2.4.

3.1.2.6. Plant cultivation

To maximize the number of cuttings per experimental group, hydroponic solutions were chosen. Poplar cuttings (*Populus deltoides x* (*trichocarpa x deltoides*)) of 30 cm were grown in aerated tap water till sufficient roots were formed. Thereafter, cuttings were transferred to a hydroponic system (2 liter, aerated solution) with or without inoculums (see section 3.1.2.7). Control cuttings were growing in ½ Hoagland's solution (Hoagland and Arnon, 1938), Cd-exposed cuttings grew in ½ Hoagland's solution supplemented with 50 µM $CdSO_4$. For the next 10 weeks, cuttings were watered three times a week with destilled water and once per week cultures were refreshed with ½ strength Hoagland's solution with or without Cd.

3.1.2.7. Inoculation of engineered PGPB

Transconjugant strains were cultivated in liquid rich medium at 30°C. Bacterial cells were collected by centrifugation (4000 rpm, 15 min, RT), washed twice in 10 mM $MgSO_4$ and subsequently resuspended in 10 mM $MgSO_4$ (OD = 1, λ =

660). Inocula were added to ½ Hoagland's solution to a final concentration of 10^8 CFU ml^{-1}. Non-inoculated cuttings received an equal amount of 10 mM $MgSO_4$ without bacteria. After 10 weeks of hydroponic growth, poplar plants were harvested. To estimate the effects on growth, leaves, roots, shoots and cuttings were weighted and in addition, numbers of leaves were counted and shoot and root length were measured. Leaf, shoot and root samples were taken to determine Cd concentration by ICP-OES.

3.1.2.8. Statistical analysis

Datasets were analyzed using a one way or two way ANOVA (α = 0.05) and *post hoc* multiple comparison testing (Tukey Kramer) to determine statistically significant differences between groups. To approximate normality and/or homoscedasticity, transformations were applied when necessary. All statistical analysis were performed using SAS 9.2 software.

3.1.3. Results

3.1.3.1. Conjugation

Prior to conjugation, all parental strains were tested on solid rich and 284 medium, supplemented with 0, 0.4 or 0.8 mM $CdSO_4$. Since helper strain and donor strains are unable to grow on 284 medium and acceptor strains are unable to grow at a Cd concentration of 0.8 mM in 284 medium, 284 medium supplemented with 0.8 mM $CdSO_4$ was chosen to select transconjugant strains after conjugation (Table 3.1.3). Conjugation of *P. putida* W619 was more straightforward than conjugation of *Enterobacter* sp. 638, resulting in a much higher amount of *P. putida* W619-transconjugant strains compared to those of *Enterobacter* sp. 638.

Table 3.1.3: Growth capacity on different media. + indicated good bacterial growth, +/- indicates minor bacterial growth and – indicates no bacterial growth.

Growth medium	Acceptors		Helper	Donors				
	Enterobacter sp. 638	*P. putida* W619	H12	CM 1600	CM 1601	CM 1602	CM 1603	CM 1633
284	+	+	-	-	-	-	-	-
284*0.4 mM Cd	+/-	-	-	-	-	-	-	-
284*0.8 mM Cd	-	-	-	-	-	-	-	-
869	+	+	+	+	+	+	+	+
869*0.4 mM Cd	+	-	+	+	+	+	+	+
869*0.8 mM Cd	+/-	-	+	+	+	+	+	+

3.1.3.2. Phenotypical and genotypical characterization

Next, all transconjugant strains (Table 3.1.4) and parental strains (Table 3.1.5) were tested for Cd resistance on solid 284 medium supplemented with $CdSO_4$ ranging from 0.4 up to 4.0 mM and subsequently for their IAA, organic acid and siderophore production capacity. In general, conjugation succeeded better using *P. putida* W619 as acceptor strain. Transconjugant strains of *Enterobacter* sp. 638 only showed Cd resistance up to 0.4 mM $CdSO_4$, whereas transconjugant strains of *P. putida* W619 exhibited resistance up to 4.0 mM $CdSO_4$. A minor group of transconjugant strains of *P. putida* W619 were able to produce IAA and organic acid, whereas all transconjugant strains of *P. putida* W619 produced siderophores. Transconjugant strains of *Enterobacter* sp. 638 could produce siderophores and organic acid, however they were not able to produce IAA. Subsequently, transconjugant strains were tested for plasmid stability and appeared to show the same level of resistance after 100 generations (data not shown).

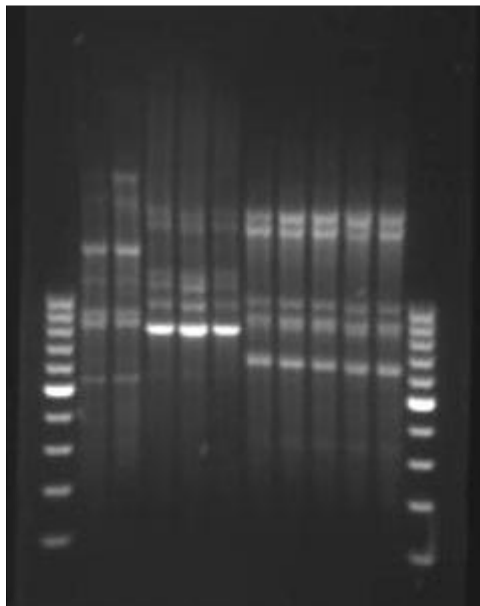


Figure 3.1.2: 1D gel-ectrophoresis analysis of BOX – PCR DNA fingerprinting. Loading scheme: 1 kb DNA ladder (GeneRuler^{TM}) – *Enterobacter* sp. 638 – E1600-11 – *P. putida* W619 – W1603-6 – W1366-5 – CM1600 – CM1601 – CM1602 – CM1603 – CM1366 - 1 kb DNA ladder (GeneRuler^{TM}).

Using BOX – PCR DNA fingerprinting analysis, bacterial colonies, picked from selective 284 medium supplemented with 0.8 mM $CdSO_4$, appeared identical to their complementary acceptor strain (Figure 3.1.2; data only shown for transconjugant strains used in greenhouse experiment). Together with the Cd resistance, this data clearly indicate the transfer of the CZR operon into the plant growth-promoting bacteria *P. putida* W619 and *Enterobacter* sp. 638.

Due to space limitations, only 3 transconjugant strains could be selected for a greenhouse experiment. This selection was based on phenotypical characteristics of the transconjugant strains. Two *P. putida* W619 transconjugant strains (W1603-6 and W1366-5) and one *Enterobacter* sp. 638 transconjugant strain (E1600-11) were chosen for the *in planta* experiment. Transconjugant strain E1600-11 was chosen since it was the only transconjugant strain of *Enterobacter* sp. 638 with a moderate Cd resistance. Transconjugant strains W1603-6 and W1366-5 were selected based on their high Cd resistance and their potential growth-promoting characteristics. The phenotypical characteristics of these stains are listed in Table 3.1.5. Since production of ACC deaminase might provide beneficial effects on plant growth, these transconjugant strains and parental strains were additionally tested for their ability to produce ACC deaminase. All strains tested positive for the production of ACC deaminase.

Table 3.1.4: Phenotypical traits of transconjugant strains; expressed in percentage of total. IAA: indole acetic acid, SID: siderophores, OA: organic acid.

Strain	Conjugation	Cadmium resistance (concentration in mM)						IAA	SID	OA
		0.4	**0.8**	**1.2**	**1.6**	**2.0**	**4.0**			
P. putida W619 with pMOL888	*P. putida* W619*H12*CM1600 *P. putida* W619*H12*CM1601 *P. putida* W619*H12*CM1602 *P. putida* W619*H12*CM1603	98.9	84.8	73.9	72.8	68.5	27.2	9.8	100.0	50.0
P. putida W619 with pMOL864	*P. putida* W619*H12*CM1366	100.0	85.7	76.2	61.9	61.9	19.0	14.3	100.0	28.6
Enterobacter sp. 638 with pMOL888	*Enterobacter* sp. 638*H12*CM1600 *Enterobacter* sp. 638*H12*CM1601 *Enterobacter* sp. 638*H12*CM1602 *Enterobacter* sp. 638*H12*CM1603	36.4	0.0	0.0	0.0	0.0	0.0	0.0	100.0	100.0
Enterobacter sp. 638 with pMOL864	*Enterobacter* sp. 638*H12*CM1366	100.0	0.0	0.0	0.0	0.0	0.0	0.0	100.0	100.0

Table 3.1.5: Phenotypical characteristics of parent strains and transconjugants selected for a greenhouse experiment. + : positive evaluation, - : negative evaluation, +/- : moderate positive evaluation, NT : not tested. IAA: indole acetic acid, SID: siderophores, OA: organic acid.

Bacterial strain	**Conjugative background**	**Cadmium resistance (concentration in mM)**							**IAA**	**OA**	**SID**	**ACC-D**
		0.2	**0.4**	**0.8**	**1.2**	**1.6**	**2.0**	**4.0**				
Parent strains												
CM1600	Donor strain	+	+	+	+/-	-	-	-	+	-	+	+
CM1601	Donor strain	+	+	+	+/-	-	-	-	+	-	+	+
CM1602	Donor strain	+	+	+	+/-	-	-	-	+	-	+	+
CM1603	Donor strain	+	+	+	+/-	-	-	-	+	-	+	+
CM1366	Donor strain	+	+	+	-	-	-	-	+	-	+	+
H12	Helper strain	+	+	+	-	-	-	-	NT	NT	NT	NT
P. putida W619	Acceptor strain	-	-	-	-	-	-	-	+	+	+	+
Enterobacter sp 638	Acceptor strain	+	+/-	-	-	-	-	-	+	+	-	+
Transconjugants												
E1600-11	*Enterobacter* sp. 638 * H12 * CM1600	+	-	-	-	-	-	-	-	+	+	+
W1603-6	*P. putida* W619 * H12 * CM1603	+	+	+	+	+	+	+/-	-	+	+	+
W1366-5	*P. putida* W619 * H12 * CM1366	+	+	+	+	+	+	-	-	-	+	+

3.1.3.3. Cd extraction from liquid medium

Since bacteria, possessing the CZR operon, are believed to precipitate Cd on their cell wall, the selected transconjugant strains were tested for their ability to extract Cd from liquid 284 medium (Figure 3.1.3). Significant differences in Cd removal were found between *P. putida* W619 and transconjugant strain W1366-5, indicating that equipping *P. putida* W619 with the CZR operon enables the bacteria to extract more Cd from the medium. Transconjugant strain W1603-6 showed an increasing trend in Cd removal, however no significant difference present compared to the parental strain *P. putida* W619. The same pattern was observed for transconjugant strain E1600-11, however the increase was not significantly different from the parental strain *Enterobacter* sp. 638.

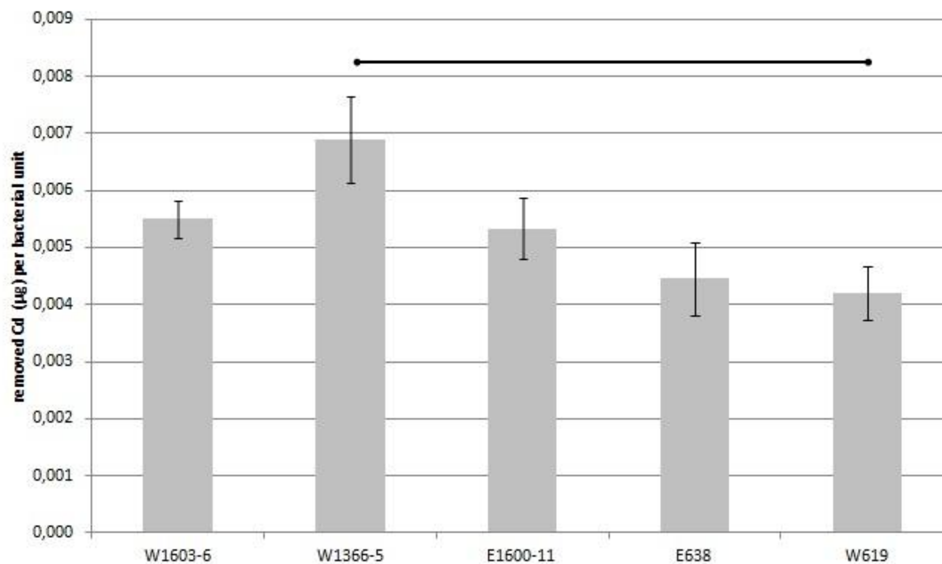


Figure 3.1.3: Cadmium (µg) extracted from the medium after 2 days of bacterial growth. ●—● indicates significant differences (two way ANOVA, $p<0.05$). No Cd was measured in Cd free media, independent of the bacterial strains and were therefore excluded from the graph.

Since the CZR operon works as an antiport mechanism where 2 protons are imported for each exported Cd molecule (Nies *et al.*, 1989), pH in the medium is expected to increase when the CZR mechanism is active. Since the difference in pH is calculated as $\frac{pHT0-pHTx}{pHT0}$, with pHT0 the pH measured on time point zero and pHTx the pH measured after 4 days of growth, an increase in pH is indicated as

a negative value (Figure 3.1.4). Since bacteria are able to produce other components that can have an influence on the pH, the effect on pH induced in control medium was verified for all bacterial strains.

For all donor strains, a decreasing trend in pH was present when grown in Cd supplemented medium. Acceptor strain *Enterobacter* sp. 638 induces a significant increase in pH when grown in the presence of Cd. When equipped with the CZR operon (strain E1600-11), this increase in pH is lower in Cd medium compared to the parental strain, but still significantly higher compared to control medium. Parental strain *P. putida* W619 induces an increasing trend in pH in control medium but a decreasing trend in Cd supplemented medium. Equipped with the CZR operon, *P. putida* W619 transconjugant strains (W1603-6 and W1366-5) behave differently. Although not significant compared to its parental strain, a pH decrease was present for strain W1603-6 in both control and Cd supplemented medium. For transconjugant strain W1366-5 a decreasing trend in pH was present in control medium while an increasing trend in pH was present in Cd supplemented medium, compared to the parental strain. However, the described differences between control and Cd supplemented medium are not significant.

Compared to its donor, transconjugant strain W1603-6 induces a lower pH decrease which is significant in Cd supplemented medium. Transconjugant strains W1366-5 and E1600-11 significantly lower the pH decrease induced by their donors in control medium. In Cd supplemented media both transconjugant strains increase the pH after 4 days of growth. These increases are significantly different from the decreased pH induced by their donors in Cd supplemented media. These data suggest that the CZR operon is not active in the donor strains since the pH is slightly decreased when Cd is present.

The significant difference induced by transconjugant strain E1600-11 however is probably not caused by the presence of the CZR operon since its parental strain *Enterobacter* sp.638 induces a similar pH increase upon Cd exposure. Although not significant, transconjugant strain W1366-5 increases the pH when grown in Cd supplemented medium while its parental strain lowers the pH, suggesting that equipping *P. putida* W619 with the CZR operon resulted in a transconjugant strain with an active CZR operon upon Cd exposure.

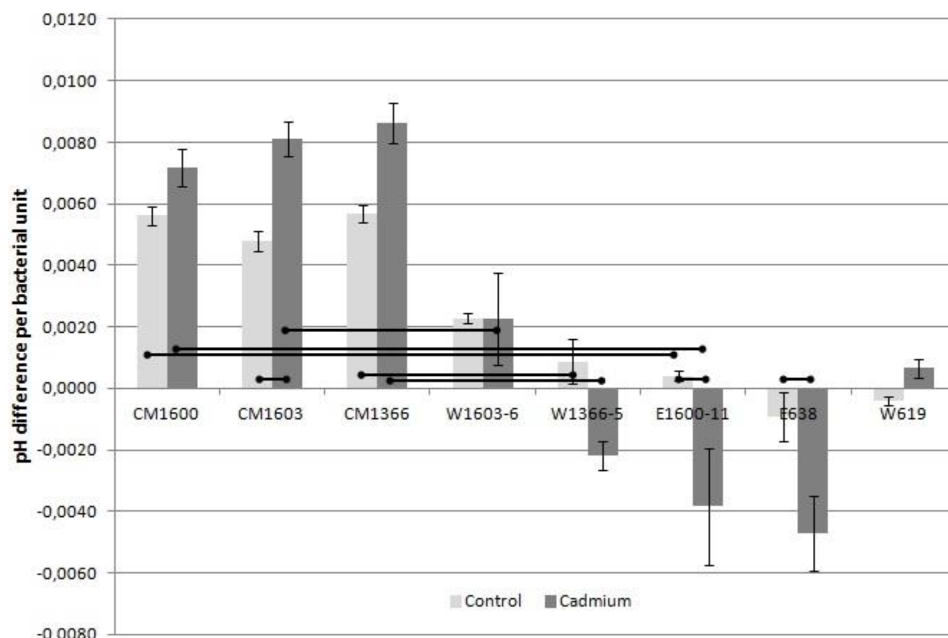


Figure 3.1.4: pH difference measured in the medium after 4 days of bacterial growth. •—• indicates significant differences among the groups (two way ANOVA, $p<0.05$).

3.1.3.4. Cd (im)mobilization from contaminated soil

Based on their results, Kuffner *et al.* (2010) hypothesized that the ability of bacterial strains to mobilize or immobilize Cd from contaminated soil, might provide a straight indication for their behaviour *in planta*. Therefore, the selected transconjugant strains were tested for their ability to (im)mobilize Cd from contaminated soil. Soil was obtained from a test field in Lommel, known to be contaminated with 5 mg Cd kg^{-1} DW soil. Since bacteria were removed from the growth medium prior to soil addition, Cd could not precipitate on the bacterial cells. By consequence, only the effect of bacterial exudates on Cd (im)mobilization in the soil was measured. In control samples, here defined as samples in which no bacteria were grown prior to addition of contaminated soil, a higher Cd concentration was present compared to the exposed samples, here defined as samples in which bacterial cells were grown prior to addition of contaminated soil (Figure 3.1.5A), indicating that bacterial exudates immobilized Cd in the soil. To compare the influence of the bacterial strains, total Cd

concentration in the medium gives a dubious representation of the Cd (im)mobilization since it does not take the amount of bacteria into account. Based on the amount of Cd removed per bacterial unit, strain W1366-5 seemed to immobilize less than the other strains (Figure 3.1.5B). However, no significant differences were found between the bacterial strains (one way ANOVA, $\alpha<0.05$). Based on the phenotypic traits of the transconjugant strains, these results are somewhat unexpected since all 3 strains showed siderophore production and strains E1600-11 and W1603-6 are also able to produce organic acids (Table 3.1.5). However, it is not sure that the life time of siderophores and organic acids is sufficient long to perform an effect, since they might be degraded by bacteria present in the soil.
To get an idea of the effect on the pH, the pH was measured on the medium solution prior to adding the contaminated soil (pHT0) and after 2 hours of contact with the contaminated soil (pHTx). Since the difference in pH is calculated as $\frac{pHT0-pHTx}{pHT0}$, a pH decrease is presented as a positive value (Figure 3.1.5C). The pH differences between time zero and after 2 hours of contact with the soil, indicate that control media became more basic, while exposed media became more acidic (Figure 3.1.5C). This could be due to the production of organic acid, however the greatest difference was described for transconjugant strain W1366-5 which tested negative for organic acid production. Since the transconjugant strains were not in direct contact with the Cd contaminated soil, precipitation of Cd onto the bacterial cell wall by the action of the CZR operon was not possible.

3.1.3.5. Effects on poplar plants: a greenhouse experiment

Poplar cuttings were inoculated with the selected transconjugant strains and grown for 10 weeks in a greenhouse. Thereafter, cuttings were harvested and growth parameters were determined to assess the *in vivo* effects of the selected transconjugant strains on poplar growth (Figure 3.1.6). Although not significant, Cd exposure generally reduced growth of *Populus deltoides x (trichocarpa x deltoides)*. Without Cd, growth of poplar cuttings shows an increasing trend when inoculated with transconjugant strains W1603-6 or W1366-5 compared to non inoculated cuttings.

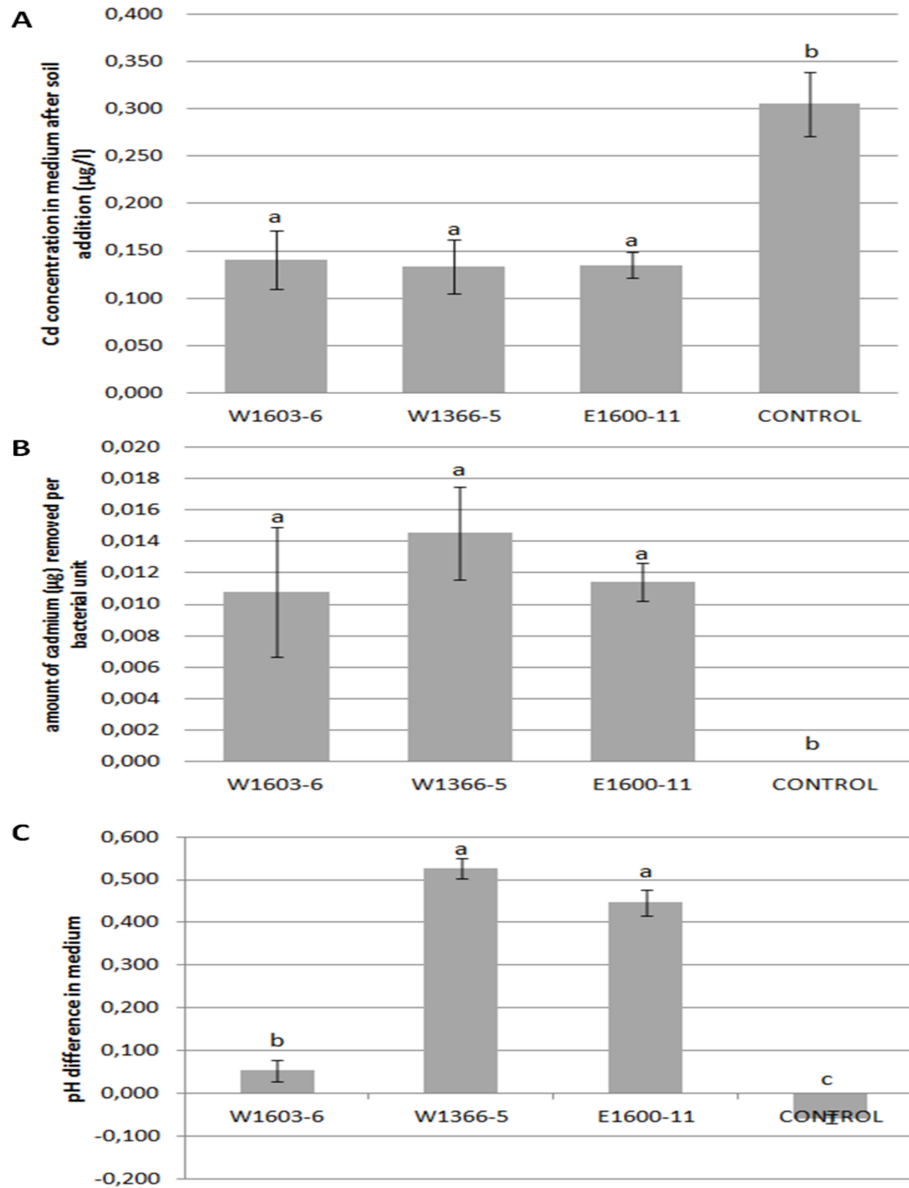


Figure 3.1.5 Cd (im)mobilization from contaminated soil. (A) Measured Cd concentration in the medium by ICP, (B) amount of Cd removed per bacterial unit; one bacterial unit represents $1*10^{-8}$ bacterial cells, (C) measured pH difference between time point zero and after 2 hours of contact with contaminated soil. Significant differences are indicated by lowercase letters, groups with the same letter are not significantly different (one way ANOVA, $\alpha<0.05$).

When exposed to Cd, the same trend was observed. After inoculating cuttings with transconjugant strain E1600-11, a different effect was present; in absence of Cd the presence of transconjugant strain E1600-11 caused a decreasing trend in plant growth. Although no significant differences were present, the decreasing trend manifested on all growth parameters. Upon Cd exposure, transconjugant strain E1600-11 significantly increased shoot length and root mass compared to non inoculated cuttings. Further, significant differences were present between root mass in control and Cd exposure when cuttings were inoculated with transconjugant strain E1600-11.

Cadmium concentration was determined in leaves, shoots and roots of inoculated and non inoculated cuttings under control and Cd-exposed conditions (Figure 3.1.7). Although no significant differences were observed compared to non inoculated cuttings, transconjugant strains W1603-6 and W1366-5 induced an increasing trend of Cd concentrations in leaves. On root level, inoculation with W1603-6 lead to a decreasing trend in Cd concentration which, together with the increasing trend in Cd concentration in leaves, suggests a more efficient translocation. Due to technical errors, data on transconjugant strain E1600-11 were lost.

To get a better idea of the amount of Cd removed after harvesting the aboveground biomass (Figure 3.1.8), this fraction is calculated as follows:

$$average\ shoot\ Cd\ concentration * average\ shoot\ DW$$

The amount of Cd that is retained in the roots (Figure 3.1.8) is calculated similarly:

$$average\ root\ Cd\ concentration * average\ root\ DW$$

Based on these amounts, a higher amount of Cd seems to be removed when cuttings are inoculated with transconjugant strain W1603-6 or W1366-5. However, the amount of Cd retained in the roots seems to increase as well after inoculation with transconjugant strain W1603-6 or W1366-5. This is due to the fact that a larger root biomass is established after inoculation with these transconjugant strains. Due to technical errors, the Cd concentration in leaves, shoots and roots could not be determined for transconjugant strain E1600-11 and subsequently no data concerning Cd removal or Cd retention could be presented for this transconjugant strain.

Figure 3.1.6: Effect of transconjugant strains on (A) weight (g) of leaves, shoot and root and on (B) shoot length (cm), root length (cm) and number of leaves. Data are presented as average ± standard error. Asterisks indicate significant differences with the non inoculated cuttings of the same exposure condition.

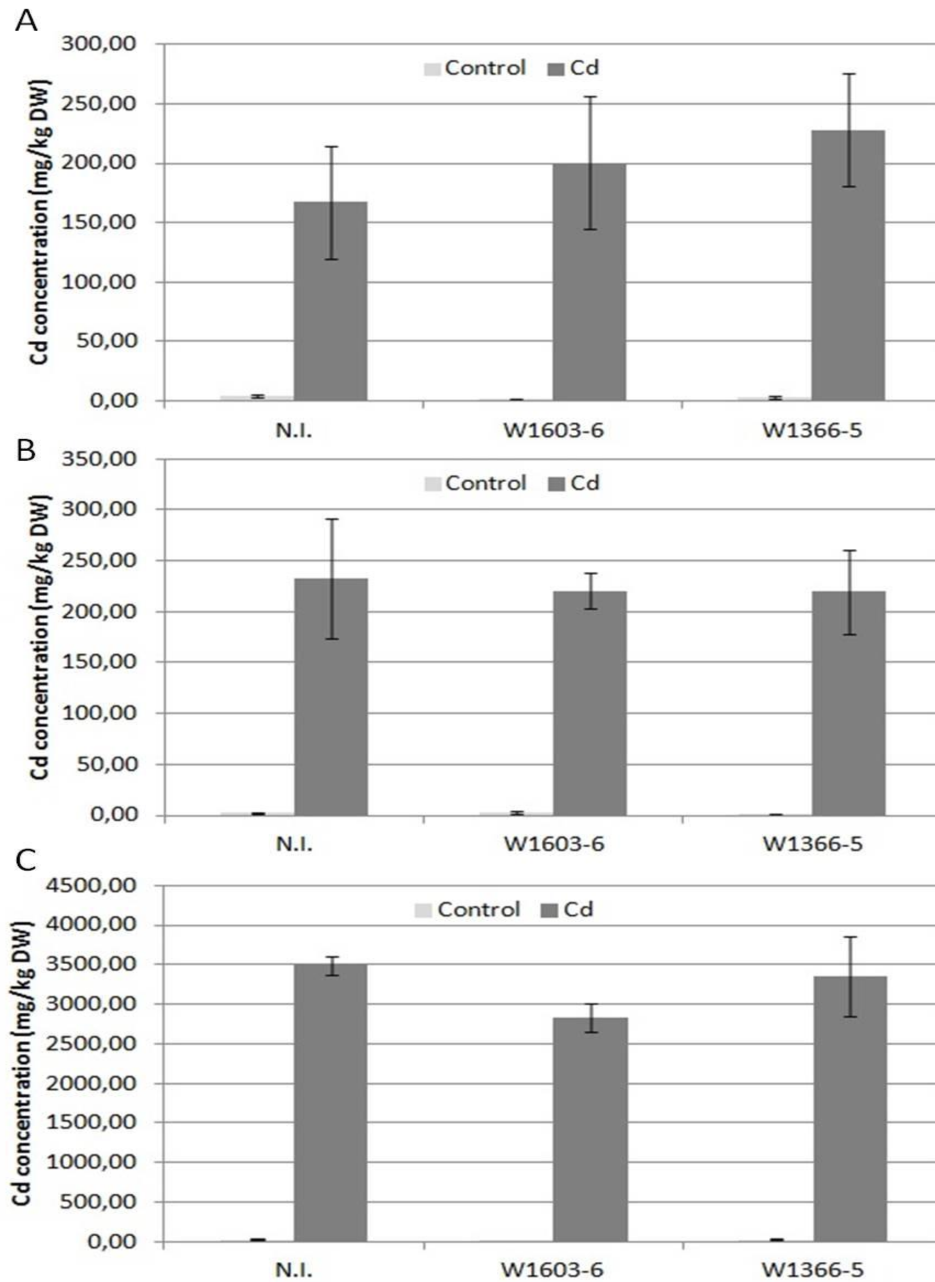


Figure 3.1.7: Effect of transconjugant strains on Cd concentration (mg kg^{-1} DW) in (A) leaves, (B) shoots and (C) roots. Data are presented as average ± standard error. Significant differences are present between control and Cd-exposed cuttings for all experimental groups. N.I.: non-inoculated.

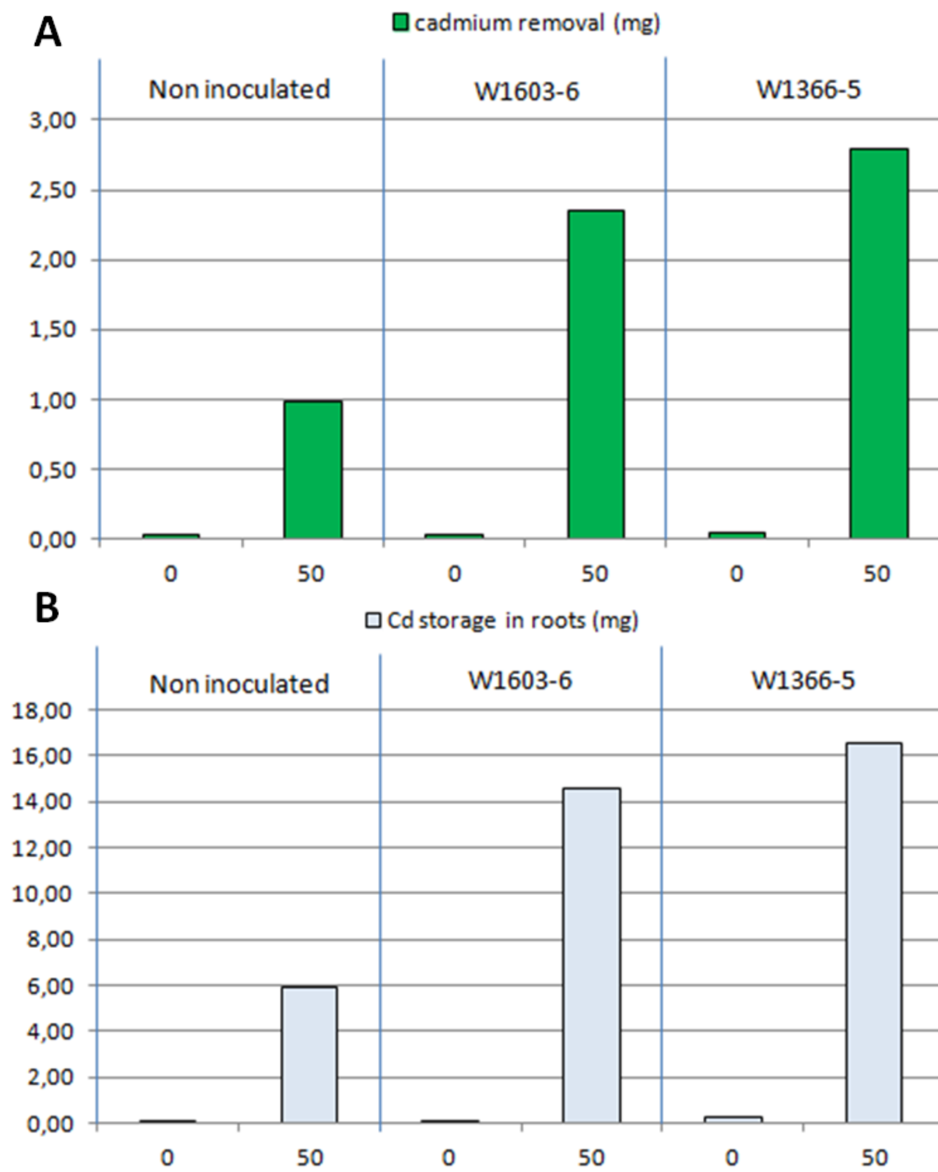


Figure 3.1.8: (A) Amount of Cd (mg) removed by harvesting aboveground biomass and (B) amount of Cd (mg) retained in roots.

3.1.4. Discussion

Phytoextraction of metals is known to be a long term process and is questioned for its ability to remove 100% of the contamination (Weyens *et al.*, 2009a; Vangronsveld *et al.*, 2009). As stand-alone technique it is often difficult to make a strong case for phytoremediation (Dickinson *et al.*, 2009). Therefore chemically assisted and bio-augmentation assisted phytoextraction gained more interest during the latest years. In case of chemically assisted phytoextraction, soil amendments are used for their ability to increase plant uptake of heavy metals, as reviewed by Meers *et al.* (2008). Bio-augmented assisted phytoextraction is defined as the use of bacteria and/or fungi to enhance metal uptake in plants and is reviewed by Lebeau *et al.* (2008). To enhance phytoremediation of organic polluted soils, engineered bacteria can be used. For instance, the inoculation of poplar trees planted on a TCE contaminated groundwater plume reduced TCE evapotranspiration by 90% (Weyens *et al.*, 2009b). Based on these and other similar findings we hypothesized that equipping plant growth-promoting bacteria (PGPBs) with the CZR operon might enhance growth and Cd uptake of poplar growing on Cd contaminated soils. Therefore two PGPBs isolated from poplar, *P. putida* W619 and *Enterobacter* sp. 638 (Taghavi *et al.*, 2005; 2009; 2010; Weyens *et al.*, 2011a) were selected to be equipped with the CZR operon via triparental conjugation. Since horizontal gene transfer is a naturally occurring process, engineered PGPBs are not in conflict with the law on GMOs, which is very strict in Belgium. Conjugation of *P. putida* W619 was quite straightforward; more than 60% of the transconjugant strains showed resistance to 2.0 mM Cd (Table 3.1.4). Conjugation of *Enterobacter* sp. 638, however, turned out to be more difficult: transconjugant strains were only resistant to 0.4 mM Cd (Table 3.1.4). Moreover, phenotypical characteristics changed after conjugation. Compared to their wild type PGPB, transconjugant strain W1366-5 lost its ability to produce IAA and organic acids (Table 3.1.5), and transconjugant strain W1603-6 and E1600-11 could no longer produce IAA. A loss of trait is often observed for bacterial strains stored under laboratory conditions (data not shown), however the ability of transconjugant strain E1600-11 to produce siderophores after conjugation was rather unexpected. A closer look to the traits of the donor strains indicates that this

might be a result of gain of trait during the triparental conjugation since donor stains were all able to produce siderophores.

Before being inoculated on poplar cuttings, transconjugant strains were tested *in vitro* for their ability to extract Cd from liquid medium. An increasing trend in Cd removal from the medium was present for all transconjugant strains. Only transconjugant strain W1366-5 realized a significant increase of Cd removal from the medium (Figure 3.1.3). Parental strains *P. putida* W619 and *Enterobacter* sp. 638 were included in the test to estimate the amount of Cd that could be removed from the medium without the presence of the CZR operon. Therefore, these increases are believed to be due to the action of the CZR operon. During this experiment, the pH of the medium decreased for all donor strains, which might indicate that the CZR operon is not expressed in these strains (Figure 3.1.4). Transconjugant strains W1366-5 and E1600-11 increased the pH of the medium upon the presence of Cd, which is another indication for the activation of the CZR operon in these transconjugant strains under Cd-exposed conditions. Transconjugant strain W1603-6 however did not induce pH increase in the medium when Cd was present. Furthermore, the significant difference induced by transconjugant strain E1600-11 is not exclusively induced by the presence of the CZR operon since its parental strain *Enterobacter* sp.638 produces a similar pH increase upon Cd exposure. Therefore, the highest pH difference, as a result of the CZR operon, is realized by transconjugant strain W1366-5. These data correlate perfectly with the abovementioned observation that only transconjugant strain W1366-5 is able to induce a significant increase in Cd removal from liquid medium (Figure 3.1.3).

To test bacteria for their ability to produce metal (im)mobilizing exudates, Kuffner *et al.* (2010) developed a protocol to extract metals from or inhibit them in contaminated soil with filtrates from liquid bacterial cultures. They reported that strain RX232 reduced the amount of Cd extracted from the medium and lowered the Cd uptake in *S. caprea* roots after inoculation. In contrast, strain EX72 enhanced Cd extraction from the medium and significantly increased Cd concentration in *S. caprea* leaves after inculation. Kuffner *et al.* (2010) concluded that these metal mobilization experiments allowed to predict effects on *S. caprea* more reliable than plant growth-promoting activity tests. Therefore, we tested transconjugant strains for their ability to remove Cd from

soils of a contaminated site in Lommel, characterized by a Cd concentration of 5 mg Cd kg^{-1} DW. In contrast to the results of Kuffner *et al.* (2010), all strains decreased pH in the medium, while it was increased in control samples, which were defined as samples in which no bacteria were grown prior to addition of contaminated soil (Figure 3.1.5A). Kuffner *et al.* (2010) speculated that the increase of extractability of Cd and/or Zn was not an effect of acidification since the medium pH rose in metal-mobilizing and metal-immobilizing cultures at the same rate. Our results however clearly indicate acidification of the medium (Figure 3.1.5c). In spite of this acidification, no Cd mobilization from the medium was present. All transconjugant strains appeared to immobilize Cd in the soil since significantly less Cd is found after soil addition compared to control samples, (Figure 3.1.5A). To facilitate comparison between bacterial strains, Cd removal was expressed per bacterial unit. Although not significantly, transconjugant strain W1366-5 immobilized less Cd per bacterial unit than transconjugant strains W1603-6 and E1600-11 (Figure 3.1.5B).

Transconjugant strains were also tested in a bio-augmentation experiment with *Populus deltoides x (trichocarpa x deltoides)* cv. Grimminge. Therefore, poplar cuttings were inoculated with transconjugant strains W1603-6, W1366-5 and E1600-11 and grown with or without the presence of Cd. After 10 weeks of growth, an increasing trend for growth was present for all Cd-exposed cuttings inoculated with transconjugant strains (Figure 3.1.6). Under control conditions, inoculation with W1603-6 and W1366-5 induced an increasing trend in growth while E1600-11 reduced growth of poplar cuttings (Figure 3.1.6). Significant growth stimulation was only present after inoculation with transconjugant strain E1600-11 (Figure 3.1.6). The Cd concentrations in leaves, shoots and roots only allowed some preliminary hypothesis. After inoculation with transconjugant strain W1366-5 and W1603-6, an increasing trend in Cd concentration in leaves was present (Figure 3.1.7). Moreover, transconjugant strain W1603-6 might be able to increase the translocation rate.

Based on the results of laboratory and greenhouse experiments, we can conclude that all transconjugant strains showed interesting traits to enhance phytoextraction by bio-augmentation. The preliminary data described in this research indicate that equipping PGPBs with the CZR operon might enhance the plants' ability to extract Cd from contaminated soils. Moreover, the

transconjugant strains were able to enhance plant growth which indirectly enhanced Cd uptake due to the 'dilution effect'; since more biomass is produced, a higher amount of Cd can be taken up before toxic effects occur. Transconjugant strain W1366-5 seems the most promising since this strain significantly extracts Cd from the medium (Figure 3.1.3) and compared to W1603-6 and E1600-11, transconjugant strain W1366-5 immobilized less Cd from the soil per bacterial unit (Figure 3.1.5). However, the data from the greenhouse experiment suggested that transconjugant strain E1600-11 is more promising under Cd-exposed conditions (Figure 3.1.6). Based on the amount of Cd removed after harvesting the aboveground biomass, transconjugant strain W1603-6 and W1366-5 seemed to enhance the fytoextraction efficiency (Figure 3.1.8). Since both transconjugant strains increase root biomass, the amount of Cd retained in the roots was increased as well (Figure 3.1.8). Subsequently, to additionally enhance the fytoextraction efficiency, it could be worthwhile to increase Cd translocation when applying transconjugant strain W1603-6 or W1366-5.

Subsequently, correlation between the Cd (im)mobilization experiment and the effect on poplar cuttings seemed not that straightforward as described for S. caprea (Kuffner *et al.*, 2010). With the objective of phytoextracting Cd contaminated soils in the North eastern part of Belgium, these transconjugant strains will be included in a future large scale and long term experiment with more replicates per experimental group to confirm these preliminary data.

Section IV:

PROTEOMICS

Chapter 4.1

A comparative study of soluble protein extractions of Populus deltoides x (trichocarpa x deltoides) for two - dimensional gel electrophoresis.

Joke Dupae, Ann Cuypers, Jean-Paul Noben, Jana Boulet, Nele Weyens, Karen Verstraelen and Jaco Vangronsveld. A comparative study of soluble protein extractions of *Populus deltoides x (trichocarpa x deltoides)* for 2-DE. *Published in Journal of Life Science, 2012,* ***6*** *(9) pp 970 – 979.*

4.1.1. Introduction

Due to their low protein content and high protease activity, proteomic analysis of plant tissues appears to be particularly challenging. Moreover, other compounds such as phenols, terpenes, organic acids, pigments, oxidative enzymes, lipids, etc. can interfere with two dimensional gel electrophoresis (2-DE) and can cause vertical or horizontal streaking, smearing or reduction in the number of distinctly resolved spots (Carpentier *et al.*, 2005; Saravanan and Rose, 2004; Wang *et al.*, 2003). Protein extraction is therefore a crucial step in two-dimensional gel electrophoresis.

Populus is a well-established model organism to elucidate the biological function unique to trees (Plomion *et al.*, 2006). The genome of *Populus trichocarpa* has been sequenced (Tuskan *et al.*, 2006), paving the way for poplar proteomics. Proteome studies on poplar have been published (Bohler *et al.*, 2007; Kieffer *et al.*, 2008; Kieffer *et al.*, 2009a), but up to date no comparative study on protein extraction of poplar has been released. As protein extraction is critical in 2-DE and since every extraction method has its limitations, advantages and disadvantages (Carpentier *et al.*, 2005), it is essential to find an optimal extraction protocol before starting an experimental set-up. Therefore the aim of this study was to optimize an extraction method for soluble proteins of poplar leaves and roots.

A literature review demonstrated that a TCA/acetone extraction, a phenol extraction or a combination of both, are often used for the extraction of soluble plant proteins. In this study, 3 protocols were selected for comparison: (1) a

combination of acetone and phenol; a slightly changed protocol based on Carpentier *et al.* (2005), (2) a combination of TCA/acetone and phenol; this protocol was proposed by Wang *et al.* (2006) to be universal, rapid and especially suited for recalcitrant plant tissues. And finally, (3) a TCA/DTT/acetone extraction; a protocol already applied on *Populus tremula* L. x *P. alba* L. (*Populus* x *canescens* (Aiton) Smith) - clone INRA 717-1-B4 and *Populus tremula* L. leaves (Bohler *et al.*, 2007; Kieffer *et al.*, 2008; Kieffer *et al.*, 2009a). By comparing these protocols and by further improving the spot pattern, an optimized extraction protocol for soluble leaf and root proteins of *Populus deltoides x (trichocarpa x deltoides)* was established, however no fundamentally novel insights were acquired. In prospect of future experiments, buffer compatibility was checked for DIGE analysis.

4.1.2. Materials and methods

4.1.2.1. Plant material

Cuttings (30 cm, with an average shoot height of 10 cm) of *Populus deltoides x (trichocarpa x deltoides)* were grown on sand in 4 l pots during 10 weeks. They were watered three times a week with ½ strength Hoagland's solution (Hoagland and Arnon, 1938), until they had sufficient expanded leaves. Of each plant 3 fully expanded leaves, developed after transfer to the soil system, were harvested. To minimize biological variation, methods were evaluated 'within leaf', meaning that every possible combination of two out of three extraction methods was performed on separate leaf halves derived from a same leaf. This design allowed for comparing the three methods within one plant. For each combination three biological replicates were performed using three plants in total. In future experiments poplar cuttings will be exposed to different exposures and proteins obtained from roots and leaves will be compared. Therefore, highest reproducible protocol was also tested for its applicability on roots as well.

4.1.2.2. Extraction methods

At first we compared the differences between extraction of soluble proteins within the same leaf during 1 h and overnight for each extraction method. Subsequently we compared the three methods to each other in a loop design and one phenol based method was included afterwards (Sarma *et al.*, 2008).

4.1.2.2.1. TCA/acetone – phenol extraction

Prior to grinding in liquid nitrogen, the primary and secondary veins of the leaves were removed. The extraction was performed according to Wang *et al.* (2006) (tested on bamboo (*Bambusa vulgaris*), grape (*Vitis vinifera*), iris (*Iris pseudacorus*), olive (*Olea europea*), lemon (*Citrus limonum*), pine (*Pinus nigra*), redwood (*Sequoia sempervirens*), sugarcane (*Saccharum officinarum*), and tobacco (*Nicotiana tabacum*)) with some modifications. Because of the much higher amount of starting material, we used 10% TCA/acetone; 80% methanol-0.1 M ammonium acetate and 80% acetone at 10 ml per gram grinded tissue, a phenol-TRIS buffer (pH 8.0, (Thiellement *et al.*, 2006) was used instead of a phenol-SDS buffer and our samples were allowed to precipitate during 1 h or overnight at -20°C. The dry pellet was finally resuspended in resuspension buffer (7 M urea, 2 M thio-urea, 4% CHAPS and 30 mM TRIS) (incubation at 18°C, 1200 rpm, 2 h (Eppendorf mixer)), centrifuged (70 000 g, 90 min, 18°C) and supernatant stored at -80°C.

4.1.2.2.2. Acetone – phenol extraction

This protocol is based on the work of Carpentier *et al.* (2005) (tested on banana (*Musa spp.*), apple (*Malus domestica*) and potato (*Solanum tuberosum*)) with some modifications. The same handlings prior to grinding were performed as described above. Plant tissue was ground in liquid nitrogen. Per gram fresh weight (FW) 10 volumes of ice cold acetone were added. After 1 h precipitation at -20°C, a centrifugation at 10 000 g during 10 minutes at 4°C was performed. Subsequently, the pellet was freeze-dried, and 10 volumes of extraction buffer (50 mM Tris pH 8.5, 25 mM Na_2EDTA, 100 mM KCl, 30% w/v sucrose, 2% β-mercapto-ethanol and 0.4 mM PMSF) were added per gram FW. Ten minutes later, the same volume of phenol was added and the solution was mixed thoroughly. After centrifugation (8000 rpm, 5 min, 4°C) the phenolic phase was collected, 5 volumes of ammonium acetate in proportion to the volume of collected phenol, were added and the sample was allowed to precipitate at -20°C

for 1 h or overnight. After centrifugation (10 000 g, 10 min, 4°C) the pellet was washed three times with ammonium acetate and once with acetone/DTT (0.2% w/v). Finally, the pellets were resuspended in resuspension buffer as described in 4.1.2.2.1.

4.1.2.2.3. TCA/DTT/acetone extraction

This protocol is based on the work of Bohler *et al.* (2007) (tested on poplar ([*Populus tremula* L. x *P. alba* L. (*Populus* x *canescens* (Aiton) Smith) - clone INRA 717-1-B4])) without modifications. As mentioned above, the same handlings prior to grinding were performed and the samples were allowed to precipitate for 1 h or overnight at -20°C. The final pellet was treated as described in 4.1.2.2.1.

4.1.2.2.4. A phenol extraction protocol rendering high resolution and reproducibility of 2-DE

The extraction was carried out as described by Sarma *et al.* (2008) (tested on soybean) with some modifications. This protocol is based on phenol extraction of soluble proteins, rendering a high resolution of 2-DE gels and a high reproducibility. Protease inhibitors were excluded from the extraction buffer since no problems with proteases were present in the previous extraction methods. The final pellet was treated as described in 4.1.2.2.1.

4.1.2.3. RubisCO interference

Addition of ampholine to the resuspension buffer caused a position shift of the RuBisCO Large Subunit (LS) in the first dimension, resulting in lower interference of RuBisCO LS in the second dimension gel (Espagne *et al.*, 2007). Comparing the buffers used, adding 0.5% ampholine pH 4-7 resulted in a lower RuBisCO resolubilization. In order to control the RuBisCO in poplar leaf extracts, 1.25% (v/v) IPG 4 – 7 NL buffer (GE Healthcare) was added to the resuspension buffer, samples were mixed (1200 rpm) for 2 h at 18°C and finally centrifuged (70.000 g, 90 min, 18°C) to remove the unsolubilized proteins.

4.1.2.4. Protein quantification

The protein concentration of each sample was determined using the RC DC protein assay kit II (BIORAD, California) using BSA (1.54 mg ml^{-1}) as the standard. This protein assay is an improved version of the Lowry assay (Lowry

et al., 1951), modified to be reducing agent compatible (RC) and detergent compatible (DC). Color development is achieved by a two step reaction: (1) a reaction between proteins and copper in an alkaline medium followed by (2) a reduction of Folin reagent by copper-exposed proteins (Lowry *et al.*, 1951). The 'Microfuge Tube Assay Protocol', provided by BIORAD, was used with a repetition of step 4 and 5. Leaf samples and root samples were ½ diluted prior to quantification and all solutions were brought to 27°C prior to use.

4.1.2.5. Two dimensional gel electrophoresis

For the IEF (isoelectric focusing), 24 cm strips with a non-linear pH gradient ranging from 3 to 10 (GE Healthcare) were used in the IPGphor system (Amersham Biosciences, Uppsala, Sweden). The strips were rehydrated overnight in rehydration buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 65 mM DTT, 0.8% v/v IPG buffers, 0.002% v/v bromophenol blue) containing proteins. The amount of proteins loaded onto the strip, depended on the used staining method (see section 2.6). IEF was carried out on an Ettan IPGphor Manifold (GE Healthcare) with the following settings: gradient step of 100 V for 3 h, constant step of 100 V for 3 h, gradient step of 500 V for 3h, constant step of 500 V for 3 h, gradient step of 1000 V for 3 h, constant step of 1000 V for 3 h, gradient step of 8000 V for 3 h and finally a constant step of 8000 V for 7 h at 20°C with a maximum current setting of 50 µA/strip. On the paper wicks at the negative electrode, 150 µl DeStreak rehydration solution (GE Healthcare) was added to reduce streaking. After the IEF, the IPG strips were equilibrated at 18°C for 15 min in equilibration buffer (75 mM Tris pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS and a trace of bromophenol blue) supplemented with 1% w/v DTT. A second equilibration step of 15 min with the same equilibration buffer, now containing 2.5% w/v iodoacetamide, was carried out afterwards. The first equilibration step provides a completely reduced state of unalkylated, denaturated proteins. Whereas, in the second step, iodoacetamide alkylates thiol groups in order to prevent reoxidation during electrophoresis. On top, the Immobiline DryStrips are saturated with the SDS buffer system, required for the second dimension separation. The IPG strips were then sealed on top of 200mm x 260mm x 1mm, 12.5% polyacrylamide gels with 0.5% agarose in SDS running

buffer. The SDS-PAGE step was performed at 15°C in Ettan Dalt II tanks (GE Healthcare) at 0.5 W per gel for 20 h.

4.1.2.6. Staining

To visualize the protein spots, silver staining (Shevchenko *et al.*, 1996) or Gel Code Blue staining (according to the manufacturer guidelines; Thermo Scientific) were performed. After staining, the gels were scanned using a flatbed scanner (CanoScan 4400F, Canon) at highest resolution. Comparing the extraction methods and testing the highest reproducible protocol on root samples, 100 µg soluble protein was loaded onto silver stained gels. For the comparison of RuBisCO focusing and the reproducibility test, Gel Code Blue staining was used (50 or 300 µg proteins, see results). Staining the gels with Gel Code Blue gives an idea about reproducibility of DIGE gels (Bohler S., personal comments).

4.1.2.7. Image analysis

Gel analysis was performed using ImageMaster 2D Platinum 5.0. For quantitative comparison, two parameters were used: the total spot number and the ratio of the number of automatically detected spots to the number of spots after manual verification. The last parameter is included to quantify background interference; the closer the ratio is to 1, the less background interference in the gel. Further on, this ratio will be referred to as the ‘background ratio’. Values are the means ± standard error.

4.1.2.8. Statistical analysis

Statistical analysis of all data was performed using SAS 9.1. All tests included 3 biological replicates. Since all data had a normal distribution, a student’s t-test was performed to determine statistically significant differences between the groups. The significance level was set at $\alpha = 0.05$.

4.1.2.9. Spot picking and identification

Selected spots were picked manually using the OMX-S *pro* pickers. The standard protocol provided by the manufacturers was used (OMX GmbH, Weβling) for destaining, reduction, alkylation and trypsine digestion. Tryptic digests were analysed by LC-ESI-MS/MS on a LCQ Classic (Thermo Electron, San Jose, CA,

USA) ion trap mass spectrometer equipped with a nano-LC column switching system as described by Dumont *et al.* (2004). MS/MS data were searched against the *Populus trichocarpa* v1.1 protein database (45555 entries) using Mascot (version 2.1; Matrix Sciences, London, U.K.) and Sequest (version 27, rev. 12 within Proteome Discoverer version 1.0; Thermo Fisher Scientific). Sequest and Mascot parent ion mass tolerance was set to 3 Da and 1.4 Da, respectively; fragment ion tolerance was 1 Da. Carbamidomethylation of cysteine and oxidation of methionine, tryptophan and histidine were set as fixed and variable modifications, respectively. Maximally one missed cleavage was allowed. Additional information (e.g. peptide sequence, charge state of each peptide) will be provided in supplementary data.
Resulting peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (2002) within Scaffold version 2_05_02 (Proteome Software Inc., Portland, OR). Protein identifications were accepted if they could be established at greater than 99.9% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm within Scaffold (2003). To assign a function, the accepted protein identifications were searched for homology against NCBI non-redundant database (*Populus*; taxid3689). For additional information concerning the identification we refer to the supplementary data.

4.1.3. Results and discussion

In order to find an optimal protocol for the extraction of soluble proteins from poplar leaves, a comparison was made between three extraction methods: (1) a combination of acetone and phenol, (2) a combination of TCA/acetone and phenol and (3) a TCA/DTT/acetone extraction. At first, the extraction time was optimized. Samples precipitated overnight resulted in better spot patterns and less background interference than samples precipitated during one hour (data not shown). Subsequently, the three extraction methods were compared within the leaves in a loop design. Since the total spot number was discernibly lower after the TCA/acetone - phenol extraction, no further analysis was performed at this stage and the TCA/acetone - phenol extraction was excluded from further comparison (Figure 4.1.1).

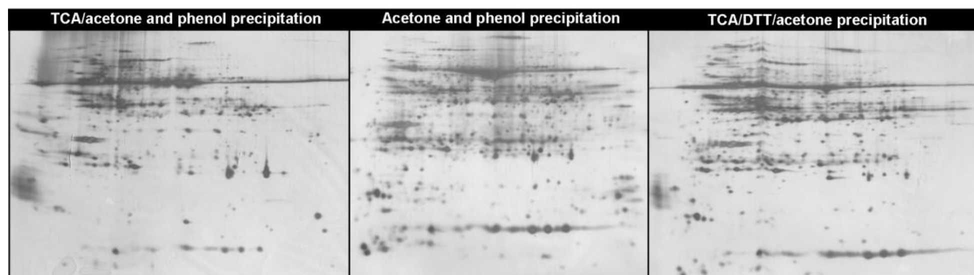


Figure 4.1.1: Comparison of the three extraction methods within the poplar leaves in a loop design; spots are revealed by silver staining. Extraction with TCA/acetone – phenol clearly rendered fewer spots and an inferior spot pattern. Therefore it was excluded from further analysis.

A new comparison between acetone - phenol and TCA/DTT/acetone was made and subjected to further analysis (Figure 4.1.2). However the TCA/DTT/acetone extraction (average total spot number: 606 ± 41; average background ratio: 0.759 ± 0.07) was used on poplar before, the acetone - phenol extraction appeared to be the best with an average total spot number of 983 ± 53 and an average background ratio of 0.905 ± 0.02. Furthermore, there was a significant statistical difference between both extraction methods for the spot number (P = 0.0004) and the background ratio (P = 0.0258). Leaves of *Populus tremula* L. x *P. alba* L. (*Populus* x *canescens* (Aiton) Smith) - clone INRA 717-1-B4 and of *Populus tremula* L., used by Bohler *et al.* (2007) and Kieffer *et al.* (2008; 2009a) respectively, are morphologically different (e.g. much thinner) compared to our poplar clone (Bohler S., personal comments). Therefore the observed difference in protein extraction capacity between these leaf types and ours, could be explained by the difference in leaf composition and structure.

Sarma *et al.* (2008) reported that they developed a phenol extraction that renders a high resolution and reproducibility of 2-DE. Using this extraction method, a similar spot pattern was obtained compared to the acetone – phenol extraction (Figure 4.1.3). No significant difference was found in the average background ratio (P = 0.7673), but the average total spot number was significantly higher with the acetone – phenol extraction (P = 0.0313).

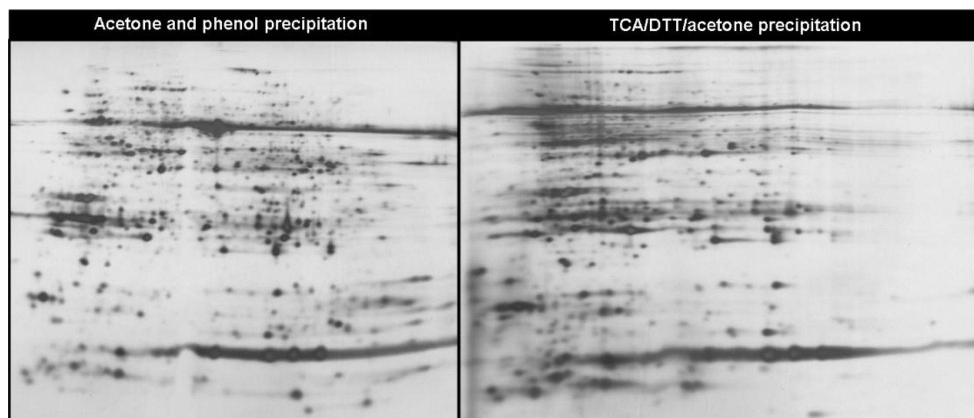


Figure 4.1.2: Comparison between acetone – phenol and TCA/DTT/acetone extraction; spots are revealed by silver staining. The acetone – phenol extraction (average total spot number of 983 ± 53, average background ratio of 0.905 ± 0.02) renders clearer spot patterns than the TCA/DTT/acetone extraction (average spot number of 606 ± 41, average background ratio of 0.759 ± 0.07)

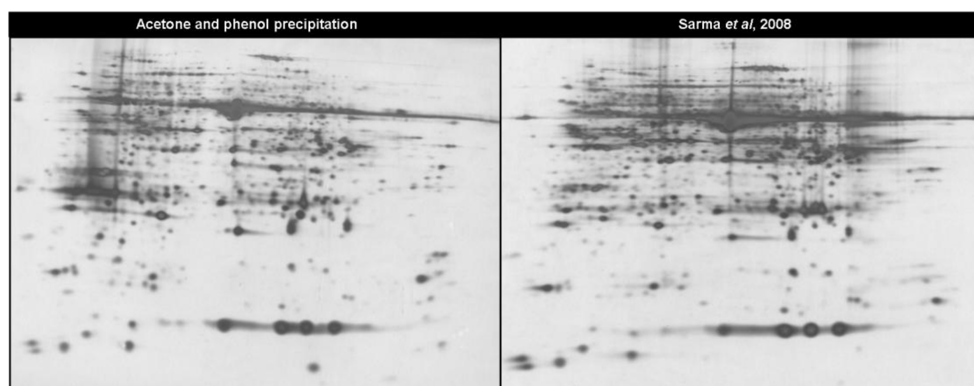


Figure 4.1.3: Comparison of the extraction proposed by Sarma *et al.* (2008) and the acetone – phenol extraction; spots are revealed by silver staining. Although a similar spot pattern was obtained using both extraction methods, the average total spot number was significantly higher with the acetone – phenol extraction (P = 0.0313). No significant differences were found between the average background ratios

Based on the former results, acetone – phenol extraction was chosen as a proper extraction method for poplar leaves and further tests were performed in order to optimize the spot pattern. As suggested by Thiellement *et al.* (2006), the pH of the phenol should always be at 8.0 to prevent nucleotide interference.

In preliminary experiments, we observed that buffers made only one day in advance (determined in the manuscript as 'fresh buffers' render clearer gels (data not shown). Additionally, calculating the volumes of all solutions in proportion to the fresh weight of the starting material, enhances the spot pattern (data not shown). Adding ampholine pH 4-7 to the resuspension buffer, Espagne *et al.* (2007) reported a position shift of the RuBisCO LS. However, adding ampholine pH 3-10, led to a selective focusing of RuBisCO LS to its correct pI. They suggested that the different mobility shift resulted from the poor RuBisCO solubilization in the 4-7 ampholine pH range. Besides the position shift of the RuBisCO LS, distinctly separated spots in the RuBisCO LS region were obtained. Moreover, some of these spots had never been identified in the plant proteome before. These results raised the question whether addition of IPG 4-7 NL buffer (GE Healthcare) to our resuspension buffer, could result in a better focused RuBisCO LS region since less RuBisCO LS is solubilized. Therefore, proteins were resuspended in the presence of 1.25 % (v/v) IPG 4-7 NL buffer (GE Healthcare) and centrifuged at 70.000 g during 90 min prior to loading. First, testing the Gel Code Blue staining, 50 µg proteins was loaded, resulting in a better focused RuBisCO LS spot (Figure 4.1.4a). The protein amount was increased up to 300 µg to obtain the same spot pattern as on silver stained gels. Loading 300 µg proteins, the RuBisCO spot is less focused but still better than without IPG 4-7 NL buffer (Figure 4.1.4b - c).

The presence of RuBisCO LS often interferes with the identification of spots with the same molecular mass or the same isoelectric point. To determine the RuBisCO LS interference, four spots (indicated by numbered arrows on figure 4.1.5) were picked for LCQ analysis. Three spots could be identified (Table 4.1.1) as (1) an ATP synthase beta subunit, (2) a fructose 1,6 bisphosphate aldolase and (3) a catalase. The same aldolase was identified in spot 4, but here, it was co-migrating with RuBisCO LS. Spot location was inconsistent with the MW of each of both proteins separately, but its occurrence was reproducible from gel to gel. The reason for this is not known. Taken together and as judged from a limited number of spots analyzed, only minor background interference might be expected coming from RuBisCO LS in planned physiological studies in poplar using the protocol as described above.

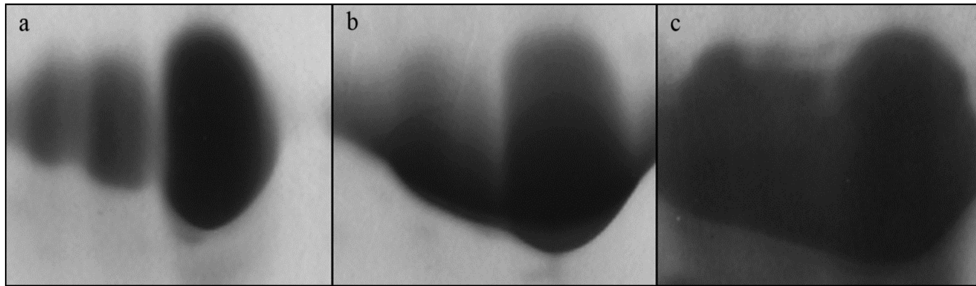


Figure 4.1.4: Focusing of RuBisCO LS region. spots are revealed by Gel Code Blue. A: 1.25% (v/v) IPG 4 – 7 NL buffer + 50 µg proteins loaded, B: 1.25% (v/v) IPG 4 – 7 NL buffer + 300 µg proteins loaded, C: no IPG 4 – 7 NL buffer + 300 µg loaded. By adding 1.25% (v/v) IPG 4 – 7 NL buffer the RuBisCO LS spot is clearly better focused

Hydrolysis of acrylamide could occur during overnight isoelectric focusing, resulting in poor focused spot patterns and sometimes even "gaps" in the basic region. Adding 150 µl DeStreak rehydration solution on the negative paper wick, hereby preventing depletion of urea and thiourea and thus diminishing hydrolysis of acrylamide (Kask *et al.*, 2009), resulted in a better spot pattern (data not shown) but could not prevent the "gaps" in the basic region (Verstraelen K, personal comments).

Differences in the average spot number were present for the acetone – phenol extraction between the different comparisons, giving doubt to the reproducibility of the extraction protocol. However, these differences could be due to the silver staining procedure, considering its low reproducibility (Görg *et al.*, 2004). To test the reproducibility of the acetone – phenol extraction, gels were stained with Gel Code Blue, which is less sensitive but more reproducible than silver staining (Görg *et al.*, 2004). Analysis of 4 gels (biological replicates) resulted in an average total spot number of 208 ± 2 and high matching percentages, suggesting a high reproducibility of the extraction method (Figure 4.1.5, Table 4.1.2).

Table 4.1.1: Protein identification to determine RuBisCO interference.

BLAST against Populus trichocarpa v1.1 protein database (45555 entries)								NCBI BLAST		
Spot number	**MW (Da)**	**Protein number - P. trichocarpa database**	**NCBI Accession number (gi)**	**Unique peptides**	**Unique spectra**	**Total spectra**	**% coverage**	**BLAST**	**Protein name**	**Query coverage**
1	53976	242776	110227086	4	4	9	7	*Populus alba*	ATP synthase beta subunit	100%
2	42832	833033	224105301	9	10	23	21	*Populus trichocarpa*	predicted protein; Fructose-1,6-bisphosphate aldolase	100%
3 (a)	42796	713130	224077927	2	2	3	6	*Populus trichocarpa*	predicted protein; Fructose-1,6-bisphosphate aldolase	100%
3 (b)	10914	270932	134093207	2	2	2	23	*Populus trichocarpa*	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	100%
4	56640	550767	224065685	2	2	2	4	*Populus trichocarpa*	catalase	100%

Spot number corresponds to the spots indicated in figure 4.1.5. Parameters obtained from BLAST against *Populus trichocarpa* v1.1 protein database (from left to right): the theoretical molecular weight (MW), protein identification number as entered in the database, number of unique peptides matched to obtain the protein sequence, number of unique spectra used in identification, the total number of spectra, the percent of coverage of the sequence obtained by mass spectrometry. Information obtained by search against NCBInr; *Populus* taxid 3689 database; (from left to right): the species to which the highest homology is found, the protein identification and percent homology found between the identified protein using *P. trichocarpa* v1.1 protein database and the identified protein using NCBInr; *Populus* taxid 3689 database (percent query coverage)

Table 4.1.2: Matching results of the gel from the reproducibility test.

GelName1	GelName2	# Matches	Percent Matches
L9	L12	198	95.0
L10	L12	200	94.8
L11	L12	194	93.3

L12 was set as reference gel. # Matches: the number of spots that could be matched between the two specified gels (GelName1 and 2), Percent Matches: percentage of spots of GelName1 that could be matched to the reference gel (GelName2).

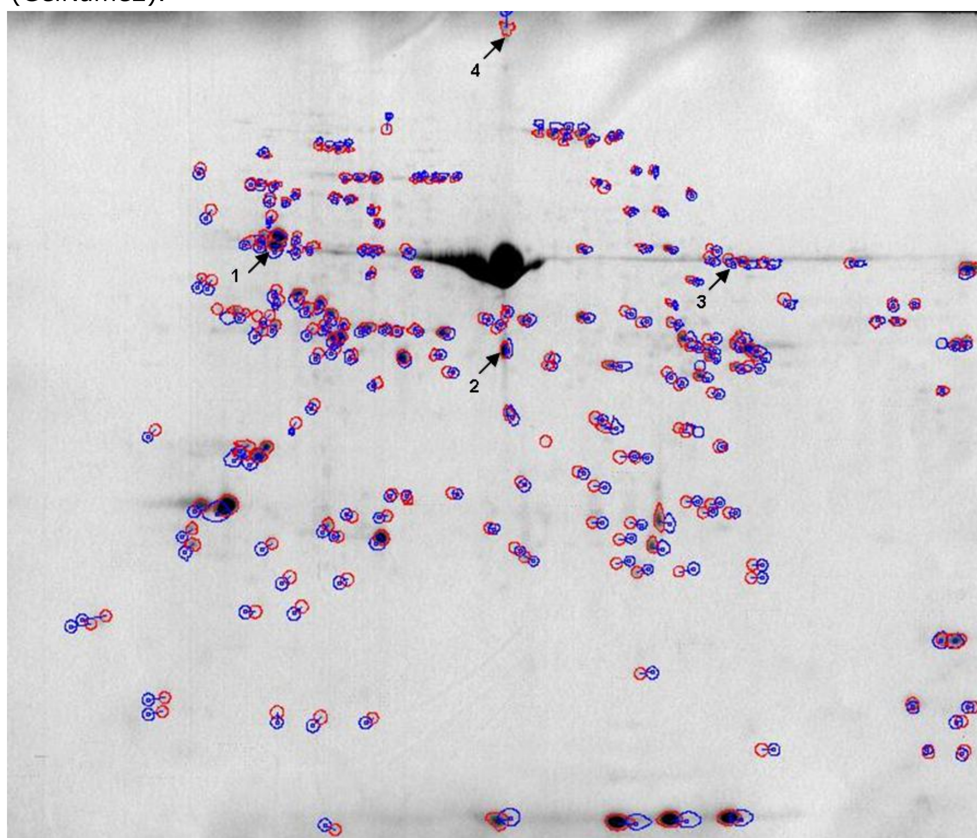


Figure 4.1.5: Acetone – phenol extraction of leaf samples. Gels were stained with Gel Code Blue and 300 µg proteins were loaded. After matching, a high reproducibility was present (Table 4.1.2). The reference gel is shown with the spot pairs; red circles represent spots belonging to the reference gel (L12), blue circles represent spots belonging to gel L10. Numbered arrows indicate the spots that were picked for LCQ analysis.

After optimizing the extraction method on leaf samples, the same protocol was used for root samples. As shown (Figure 4.1.6), this rendered very clear spot patterns with an average total spot number of 1611 ± 121 and an average background ratio of 1.03 ± 0.07.

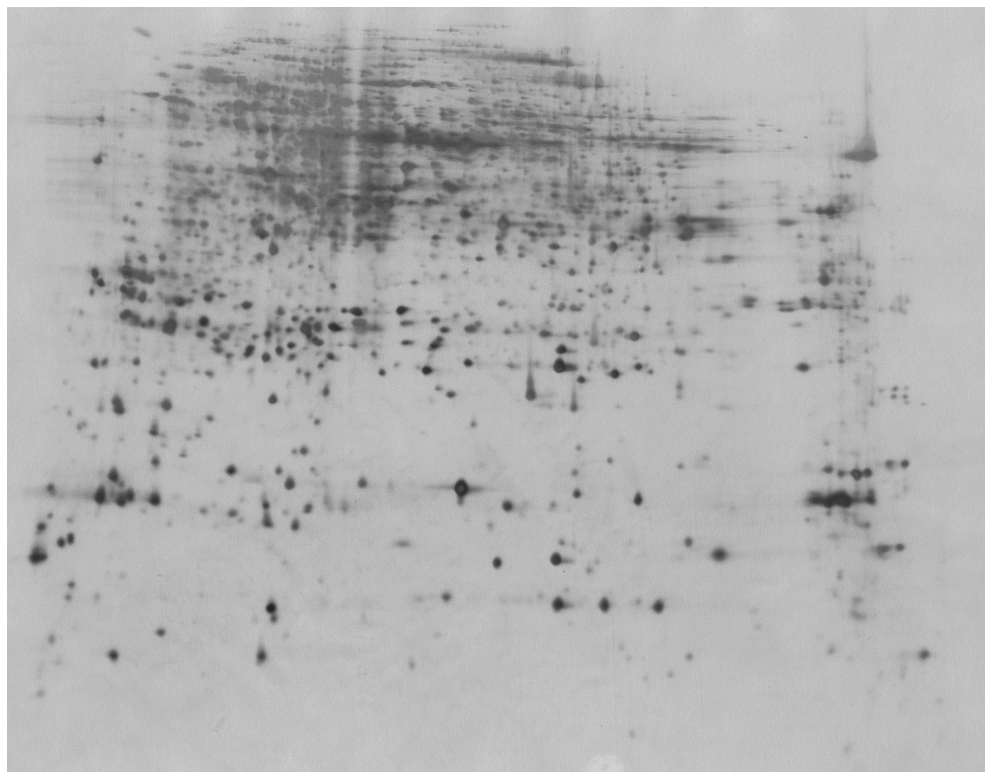


Figure 4.1.6: Acetone – phenol extraction of poplar root samples; silver staining. This extraction method provides very clear spot patterns of soluble root proteins (average total spot number of 1611 ± 121 and an average background ratio of 1.03 ± 0.07)

4.1.4. Conclusion

According to the data presented above, the acetone – phenol extraction is the highest reproducible extraction method for soluble proteins of poplar leaves, yielding a high spot number and low background interference. Applying the extraction method on root samples rendered very clear spot patterns as well. Further optimization revealed several critical steps: (1) pH of the phenol should always be at 8.0 to assure that nucleotides will be in the buffer phase and not in the phenol-rich phase (Thiellement *et al.*, 2006), (2) the extraction buffer should

always be made fresh and (3) the volumes of buffers, phenol, … added should be carefully calculated in proportion to the fresh weight of the starting material.
Some more tests made to improve the quality of the spot pattern, revealed that applying 150 µl DeStreak rehydration solution (GE Healthcare) on the paper wick of the negative electrode increases the quality of the spot pattern. On top, addition of 1.25 % (v/v) IPG 4-7 NL buffer (GE Healthcare) to the buffer prior to resuspension results in a better focused RuBisCO LS region. It should be mentioned however that, when performing DIGE, ampholytes will compete with proteins to bind the fluorophores, therefore IPG buffer may not be added prior to DIGE labelling. Scientists focusing on the RuBisCO region could use this method combined with other commercial dyes which are sensitive and can be used quantitatively.

Chapter 4.2

Problems inherent to a meta-analysis of proteomics data: A case study on the plants' response to Cd in different cultivation conditions

The first week of my PhD, a colleague told me "beware of what you're starting with". Back then, I thought I did… only now I understand I did not. Her statement was the motivation to write this chapter (With special thanks to Karen Verstraelen).

4.2.1. Introduction

Since Cd is a naturally occurring chemical element in the earth's crust, it is present in the environment at low concentrations. Because of industrial pollution in the late 19th and early 20th century, however, Cd concentrations raised in the environment. To date, 25 thousand tons Cd per year are released into the environment worldwide (WHO, 2000). Half of this amount is due to natural processes such as erosion, wood fires and volcanic eruptions. The other half is due to antropogenic pollution via recovery of iron (Fe), steel and non-ferro metals like zinc (Zn), lead (Pb) and copper (Cu), and the use of fossil fuels and phosphate based fertilizers (WHO, 2000). In Belgium, hot spots are found at sites of former Zn smelters. However, since Cd is also transported by air, Belgium copes with large scale Cd contaminated areas.
Although Cd^{2+} can substitute Zn^{2+} as a cofactor in carbonic anhydrase in marine algae (Lane *et al.*, 2000), Cd is classified as a non-essential metal and listed as nr 7 at the ATSDR (Agency for Toxic Substances and Disease Registry) top 20 list of toxic substances and as carcinogenic (group I) by the IARC (International Agency for Research on Cancer). Even at low concentrations it poses severe risks for human health, animals, plants and microorganisms (see chapter 1.1 and 1.2; Doyle *et al.*, 1975; Godt *et al.*, 2006; Kirkham 2006; Huff *et al.*, 2007). Being sessile organisms, plants need to adapt to their environment. To cope with prevailing Cd levels, different mechanisms can be addressed: (1) metal binding to the cell wall, (2) active efflux, (3) compartmentalization, (4) reduced transport across the cell membrane (5) repair and protection of the plasma membrane under stress conditions and (6) chelation of Cd molecules (Prasad *et al.*, 1995; Hall, 2002; do Nascimento and Xing, 2006). In addition, the

movement of metals to the roots can be restricted by ectomycorrhizal fungi (Hall, 2002). Metal ions are taken up in two steps by plants: at first a rapid and non-specific binding of the cations to the negatively charged membrane components located at the cell surface occurs. Secondly, an energy dependent intracellular uptake of the metal ions takes place. Cadmium easily enters the plant's root system through uptake systems for essential metals like LCT1 (low affinity cation transporter), ZIP (ZRT1/IRT1-like proteins), Nramp (natural resistance-associated macrophage protein) and SLC11 (solute carrier family 11) metal transporters or Fe^{2+}, Zn^{2+} and Ca^{2+} transporters/channels with low specificity. Moreover, the hypothesis of a Cd-specific uptake system is postulated (Clemens, 2006). The Cd availability for plants is regulated by amongst others soil pH, redox potential and chloride levels (Prasad, 1995; Smolders and McLaughlin, 1996; Bingham *et al.*, 1984; Doner, 1978).

Since almost all Cd released into the air finally binds to the organic matter in the soil (Kaschl *et al.*, 2002), Cd can easily reach the food chain by the uptake through plant roots. On top, it can easily leach to groundwater (Robinson *et al.*, 2000). Since toxic metals, like Cd, can pose severe risks to human health, threshold values have been defined for agricultural soils and crops. Exceeding these threshold values and consequently loosing economic valuable soils, enhances the authorities' interest in alternative remediation strategies. Classical remediation techniques (e.g. soil washing, excavation and dumping) are expensive, destructive for soil structure and microorganisms and not applicable for large-scale contaminations (Glick, 2003; 2010; Mastretta *et al.*, 2006; Weyens *et al.*, 2009). During the past decennia, phytoextraction, which is the use of plants to extract metals from soil and groundwater, seems promising for this purpose. A major drawback of phytoremediation remains the large time scale needed to remediate the soil, therefore phytoremediation efficiency needs to be optimized. This can either be achieved chemically using soil amendments (Komarek *et al.*, 2008) or naturally by the use of plant-associated microorganisms (e.g. Zimmer *et al.*, 2009; Ryan *et al.*, 2008; Braud *et al.*, 2009; Weyens *et al.*, 2010). A basic understanding of the plant's response to environmental relevant Cd concentrations is necessary to perform research directed at enhancing phytoremediation efficiency. Whereas numerous studies

have described symptoms and responses of plants to Cd, there is still little known about the overall molecular mechanisms that are addressed.

The main aim of this review is to provide an overview of Cd responses at the proteome level in plants' leaves and roots, taking into account the different growth systems and exposure times. However, exploring the literature and retrieving the proteomic information, revealed numerous obstacles to achieve this goal. Therefore, the current review focuses on major problems encountered in a meta-analysis of proteomic responses upon Cd stress, *i.e.* experimental setup and proteomics analysis. Thereafter, future prospects concerning uniform experiments and data publication will be highlighted. Finally, the current state of Cd-induced proteomic responses in plants is discussed.

4.2.2. Diversity among experimental setup, material, methods and data analysis

To our knowledge 33 articles have used proteomic techniques to investigate plant response to Cd (Table 4.2.1). Comparing these articles, the huge diversity in experimental setup, materials and methods and data analysis is striking. Studying the literature in order to get insights in the global response of plants to Cd is challenging, since a considerable variations among the studies are present. These variations are noticeable on both, experimental setup and proteomic analysis, and the most critical steps are discussed hereafter.

4.2.2.1. Impediments with regard to cultivation methods and exposure

4.2.2.1.1. Cultivation methods: from the laboratory to the field

Since the proteome dynamically responds to changes in the environment, cultivation methods with highly controllable parameters are preferred for proteomic studies. In laboratories, **cell suspension cultures, in vitro growth on 'Murashige and Skoog'-medium and germination in Petri-dishes** are often chosen for their controlled conditions (11 out of 33 studies). These controlled conditions are easy to handle, inexpensive and guarantee reproducible samples. Alongside the aforementioned advantages, it should be kept in mind that the conditions provided by these systems poorly resemble field conditions (Figure 4.2.1). Moreover depending on the plant species used, most in vitro systems work with small juvenile plants that do not reflect conditions

more comparable to the field. Unfortunately, this entails higher variability due to less controllable conditions. **Hydroponic cultivation systems** can be thought of as "intermediate" systems and are frequently used for proteomic studies (15 out of 33 studies). They allow the germination and complete development of plants, and still provide high controllable conditions (Wójcik *et al.*, 2005; Keunen *et al.*, 2011). On top, they reduce the period of growth and the space required to conduct an experiment (Zacchini *et al.*, 2009). A major disadvantage of hydroponic cultivation systems is still the limited comparability to field conditions: no real rhizosphere can be established, anaerobic conditions are at risk, roots formed in hydroponic systems have a different morphology compared to roots formed in soils and last but not least hydroponic systems supply a steady and constant availability of nutrients which does not reflect soils systems (Smeets *et al.*, 2008b). Therefore, with the prospect to field experiments, using only hydroponic systems for fundamental research is not recommended and the use of soil systems should be incorporated. Depending on the research question hydroponics can be favoured because of their high controllability but **soil cultivation systems** come closer to field conditions (Figure 4.2.1): a rhizosphere can be established, normal roots can be formed, nutrient availability is influenced by the soil conditions and the availability of the applied metal will reach a steady state dependent on the soil conditions. To get the same toxicity response, the latter usually requires a higher metal concentration application as compared to hydroponic cultures. Soil systems are less frequently used (6 out of 33 articles), probably due to the higher variation among individual pots. Up to date, only one study used samples obtained from the **field** (Hajduch *et al.*, 2001). Since the extrapolation of results obtained from hydroponics, soil systems, etc. to the field is not straightforward, therefore it is important to investigate the potential of this extrapolation in order to get better insights in the molecular mechanisms involved in phytoremediation in field conditions. The more profound this comprehension, the easier to discover manipulations that boost phytoremediation efficiency.

Table 4.2.1: List of articles studying cadmium response in plants. C.F.N.S. : Chemical form not specified, "?" represents no response on email about used statistics for differential expression.

Author	Plant species	Tissue	Cd concentration		Exposure time	Cultivation method	Statistics for differential expression	Cell suspension etc	Hydro ponics	Soil systems	Field	Protein extraction method	Proteomic technique
			as given by author	ppm									
Included in meta-analysis													
Visioli *et al.*, 2010	*Populus nigra*	Leaf	50 µM $CdSO_4$	5,6	3 weeks	Hydroponics 1/3 HL	Student t-test		x			Crude protein extraction using a $MgSO_4$ based buffer	2D liquid chromatography (HPCF - HPRP); MALDI-LR MS
Kieffer *et al.*, 2008	*P tremula* L	Leaf	20 µM $CdSO_4$	2,24	3,7,14 days	Hydroponics 1/4 HL	Two way anova / ANCOVA		x			Soluble protein extraction: TCA/acetone	2-DE; DIGE; MALDI-MS/MS
Kieffer *et al.*, 2009a	*P tremula* L	Leaf	20 µM $CdSO_4$	2,24	14, 28, 56 days	Hydroponics 1/4 HL	Two way anova		x			Soluble protein extraction: TCA/acetone	2-DE; DIGE; MALDI-MS/MS
Semane *et al.*, 2010	*Arabidopsis thaliana*	Leaf	1 and 10 µM $CdSO_4$	0,112 and 1,12	7 days	semi-Hydroponics 1/10 HL	Mann withney rank test		x			Soluble protein extraction: TCA/acetone	2-DE; SYPRO Ruby staining; LC-ESI-MS/MS
Fagioni and Zolla, 2009a	Spinach	Leaf (basal and apical)	100 µM Cd C.F.N.S.	11,2	0,5,18 days	Hydroponics 1/2 HL	Student t-test		x			TCA / Acetone precipitation	2-DE; Blue Silver staining; Nano-RP-HPCL-ESI-MS/MS
Kieffer *et al.*, 2009b	*P tremula* L	Leaf and root	20 µM $CdSO_4$	2,24	3, 7, 14, 28, 56 days	Hydroponics 1/4 HL	Two way anova		x			TCA / Acetone precipitation	2DE DIGE; MALDI-MS/MS
Rodriguez-Celma *et al.*, 2010	*Lycopersicon esculentum* Mill cv Tres Cantos	Root	10 and 100 µM $CdCl_2$	1,12 and 11,2	10 days	Hydroculture 1/2 HL	Student t-test		x			Phenol extraction	2-DE; Coomassie Blue R-250 staining; MALDI-LIFT MS/MS

Table 4.2.1: List of articles studying cadmium response in plants. C.F.N.S. : Chemical form not specified, “?” represents no response on email about used statistics for differential expression (Continued).

Author	Plant species	Tissue	Cd concentration		Exposure time	Cultivation method	Statistics for differential expression	Cell suspension etc	Hydro ponics	Soil systems	Field	Protein extraction method	Proteomic technique
			as given by author	ppm									
Included in meta-analysis													
Roth *et al.*, 2006	*Arabidopsis thaliana*	Root	10 µM Cd^{2+} C.F.N.S.	1,12	24 hours	Hydroculture 1/10 HL	Student t-test		x			Phenol extraction	2-DE; Silver staining; MALDI-MS
Alvarez *et al.*, 2009	*Brassica juncea*	Root	250µM $CdCl_2$	28	3 days	Hydroculture MS liquid medium - germination + soil - planting/ exposure	One way anova (email)		x			Phenol extraction	2-DE; DIGE; multiplexed isobaric tagging technology (iTRAQ); nano LC-ESI-MS/MS
Durand *et al.*, 2010	*P tremula x P alba* (717-1B4 genotype)	Leaf and cambial zone	360 mg/kg DW soil C.F.N.S.	360	61 days	Soil soil cultures; sand:peat moss soil mixture (25:75 v:v, pH6,9)	Student t-test			x		TCA / Acetone precipitation	2-DE; DIGE; MALDI-MS/MS
Aloui *et al.*, 2009	*Medicago truncatula* Gaertn. Cv Jemalong J5	Root	2 mg/kg $CdSO_4$	2	3 weeks	Sterile neutral clay loam soil:sand 1:2 v/v; water + nitrogen-enriched nutrient solution	ANOVA, Tuckey's post hoc test			x		Phenol extraction	2-DE; Coomassie Blue G-250 staining; LC MS/MS
Repetto *et al.*, 2003	*P sativum* L VIR4788	Root	100 mg/kg $CdCl_2$	100	5 weeks	Prewashed sterile sand soil	ANOVA			x		Crude protein extraction using a disodiumphosphate/ citric acid buffer	2-DE; Silver staining; LC MS/MS
Zhao *et al.*, 2011	*Phytolacca americana* L	Leaf	400 µM $CdCl_2$	44,8	48 hours	Hydroponics	Student t-test (email)		x			Protein extraction based on TRIS buffer; methanol/chloroform precipitation	2-DE; Silver staining or Coomassie briljant Blue G-250 staining; MALDI-TOF-TOF ESI-MS/MS

Table 4.2.1: List of articles studying cadmium response in plants, C.F.N.S. : Chemical form not specified, "?" represents no response on email about used statistics for differential expression (Continued).

Author	Plant species	Tissue	Cd concentration (as given by author)	Exposure time	Cultivation method	Statistics for differential expression	Cell suspension etc	Hydro ponics	Soil systems	Field	Reason not to include article form meta- analysis
Articles excluded from meta-analysis											
Sobkowiak 2006	Soybean	Cell suspension culture	3,5,6,10 µM $CdCl_2$	0,24,48,72 h	Cell suspension culture	?	x				Cell suspension culture
Hradilová 2010	Flax	Cell suspension culture	0,10,50,100 µM ($Cd(NO_3)_2$)	2,8,24,48h	Cell suspension culture	Student t-test	x				Cell suspension culture
Gillet 2006	*Chlamydo monas reinhardtii*	Algae	150 µM $CdCl_2$	4-5 days	Algae culture	Students t-test (email)	x				Algae
Gianazza 2007	*Lepidium sativum*	L sativum plantlets	2,5,10,20,50,100 mg/l $CdCl_2$	72h	Plantlets on filters in Petri-dish	?	x				Plantlets on filters in Petri-dish
Farinati 2009	*Arabidopsis halleri*	Shoots	1,0mM $CdSO_4$ AND 10mM $ZnSO_4$	30 days	Perlite 1/2 HL	Student t test			x		Mixed metal contamination
Bah 2010	*Typha angustifolia*	Leaf	1mM Cd (C.F.N.S)	30 and 70 days	Agricultural (field) soil in soils	?			x		Poor quality 2-DE gels, no statistics specified
Aina 2007	*Oryza sativa* L. cv Baldo	Root	10 and 100 µM $CdSO_4$	2 weeks	Glass pots containing solid MS medium	?	x				MS solid medium
Ashan 2007	*Oryza sativa* L. cv Hwayeong	Germinated seeds without roots (4 day old seedlings)	0,2 - 1,0 mM $CdCl_2$ range	4 days	Seed germination on filters in Petri-dish	Student t-test	x				Seeds germinated on filters in Petri-dish

Table 4.2.1: List of articles studying cadmium response in plants, C.F.N.S. : Chemical form not specified, "?" represents no response on email about used statistics for differential expression (Continued).

Author	Plant species	Tissue	Cd concentration (as given by author)	Exposure time	Cultivation method	Statistics for differential expression	Cell suspension etc	Hydro ponics	Soil systems	Field	Reason not to include article form meta-analysis
Articles excluded from meta-analysis											
Lanquar, 2007	*Arabidopsis thaliana*	Cell suspension culture	100 µM (C.F.N.S.)	24 hours	Cell suspension culture	?	x				Cell suspension culture
Lee 2010	*Oryza sativa* L cv. Dongjin	Root and leaf	100 µM $CdCl_2$	24 hours	MS medium	Student t-test	x				MS solid medium
Sarry 2006	*Arabidopsis halleri*	Cell culture	200 µM $CdCl_2$	24 hours	GB5 and MS medium	Student t-test	x				MS and GB5 medium
Schneider 2009	*Hordeum vulgare* L	Tonoplast	20 and 200 µM Cd^{2+} (C.F.N.S.)	7days	Vermiculite / Luwasa hydroculture nutrient solution	Fold change (email)			x		Tonoplast study
Tuomainen 2006	*Thlaspi caerulescens*	Root and shoot	60 µM $CdSO_4$	7 days	Hydroponics	ANOVA / KRUSKAL WALLIS (assumptions ANOVA violated)		x			Comparison between 3 subspecies on Zn and Cd. No real focus on effect of only Cd on plant.
Hajduch, 2001	*Oryza sativa* L.	Leaf	250 µM $CdCl_2$	72h	Cultivation in field, leaves were cut and floated in 250 µM $CdCl_2$ solution	?				x	In vitro cadmium exposure
Fagioni 2009b	Spinach (*S. oleracea*)	Thylakoid	20, 50, 100 µM (C.F.N.S.)	0, 5, 10, 15, 30, 33 days	Hydroponics	ANOVA / post hoc: Tukey's and Scheffe's		x			Only thylakoid proteins
Cho 2006	*Physcomitrella patens*	Gametophore / protonema	100 µM $CdCl_2$	6 days	BCD medium	Fold change (email)	x				Gametophore / protonema

Table 4.2.1: List of articles studying cadmium response in plants, C.F.N.S. : Chemical form not specified, "?" Represents no response on email about used statistics for differential expression (Continued).

Author	Plant species	Tissue	Cd concentration (as given by author)	Exposure time	Cultivation method	Statistics for differential expression	Cell suspension etc	Hydro ponics	Soil systems	Field	Reason not to include article form meta-analysis
Articles excluded from meta-analysis											
Ashan 2012	Soybean (*Glycin max* L. cult Enrei en cult Harosoy)	Root / microsomal fraction	100 µM $CdCl_2$	3 days	Hydroponics	One way ANOVA + Least Significant Differences test		x			Microsomal protein fraction
Kim 2005	*Nannochloropsis oculata*	Algae	10 µM $CdCl_2$	4 days	F/2 medium	?	x				Algae
Ge 2009	*Triticum aestivum* L. cv. Yangmai 13	Leaf	0,5mM $CdCl_2$	5 days	Hydroponics 1/1 HL	Fold change (email)		x			No statistics performed, accession numbers not always specified, no link to gi numbers
Cai 2011	Rice	Leaf and root	5 µM $CdCl_2$	15 days	Hydoponics	?		x			No statistics specified

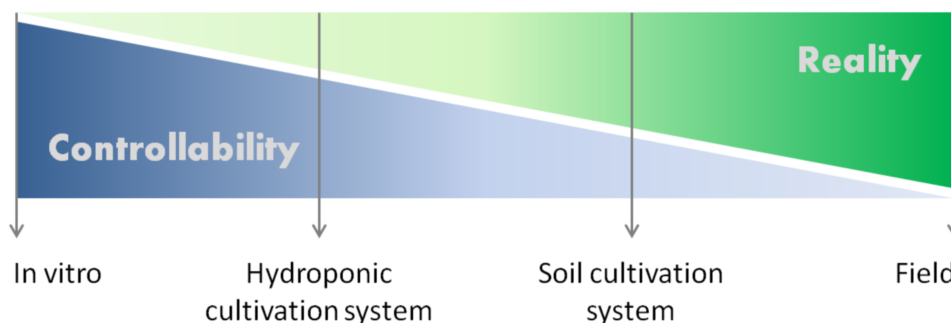


Figure 4.2.1: Differences between cultivation methods considering controllability and their ability to resemble realistic conditions.

4.2.2.1.2. The applied light conditions, temperature and relative humidity

Photosynthetic flux density used in the included articles ranges from 100 to 1000 µmol m^{-2} s^{-1} (Table 4.2.2). Moreover, large differences exist in the light periods: in the shortest period 8 hours of light were applied while in the longest period 16 hours were applied. Most studies used a light period of 16 hours for plant cultivation (Table 4.2.2). Different temperatures for growing plants were used: a range from 21 to 25°C was described. Some studies reported night temperatures ranging from 17°C to 20°C (Table 4.2.2). The maintained relative humidity is often not specified (six out of 13 articles) and ranges from 60 up to 100% (Table 4.2.2). Light conditions, temperature and relative humidity are known to have a large effect on plant growth. However, to our knowledge no study was performed revealing the exact effect of the applied light conditions and temperature and therefore, no predictions can be made considering the interaction effect of metals and environmental conditions on plant growth.

4.2.2.1.3. The applied Cd concentration

The concentration of Cd applied to the plants ranges from 1 to 250 µM and from 2 to 360 mg/kg in hydroponic and soil cultivation systems respectively. In order to create a more comparable unit, all concentrations have been recalculated to ppm. Since Cd tolerance greatly varies among plant species (Schützendübel *et al.*, 2002b), it is hard to put a general threshold to distinguish between 'low' and 'high' concentrations of Cd. Ideally, a range finding experiment should be conducted for each plant species in order to pinpoint the sub-lethal Cd concentration. Working with sublethal concentrations gives the opportunity to

study the metabolic adaptation that is required to enable plants under stress (of the toxicant) to survive and reproduce. Another manner, with regard to field experiments, is to work with environmental relevant Cd concentrations. On top, the sensitivity of plants to metals and the toxicity of metals for plants also depend on the developmental stage of the plants (Liu *et al.*, 2005). When grouping the articles, the applied Cd concentration was not taken into account. However, this will be born in mind while discussing the results.

Table 4.2.2: Light conditions applied in included research articles. If reported, temperature is specified as day/night temperature. N.S. = not specified.

Author	Photosynthetic photon flux density (µmol m^{-2} s^{-1})	Light period (hours)	Temperature (°C)	Relative humidity (%)
Visioli *et al.*, 2010	300	14	25	70-80
Kieffer *et al.*, 2008	150	16	23	N.S.
Kieffer *et al.*, 2009a	150	16	23	N.S.
Semane *et al.*, 2010	200	12	21/17	65
Fagioni and Zolla, 2009a	260 - 350	14	24/20	N.S.
Kieffer *et al.*, 2009b	150	16	23	N.S.
Rodriguez-Celma *et al.*, 2010	350	16	23/18	80
Roth *et al.*, 2006	230 - 240	8	23	N.S.
Alvarez *et al.*, 2009	100	16	24	100
Durand *et al.*, 2010	1000	16	21	70
Aloui *et al.*, 2009	220	16	23/18	60
Repetto *et al.*, 2003	N.S.	16	24/20	60
Zhao *et al.*, 2011	150	N.S.	25	N.S.

4.2.2.1.4. The chemical speciation of Cd

The chemical form in which Cd is applied differs among the included studies, making them hard to compare since each chemical form could introduce specific secondary effects. However, a distinction should be made between applying Cd as a nitrate of sulphate, which are macro-elements or as chlorine which is a micro-element. That is, using $CdSO_4$ or $Cd(NO_3)_2$, these secondary effects will only be moderate since sulphates and nitrates are present in a much higher

concentration in the nutrition solution compared to chlorine. The addition of sulphates could protect plants more against Cd toxicity since Cd induces an enhanced sulphate uptake leading to an increase of glutathione, the precursor for phytochelatins (Prasad, 1995; Hassan *et al.*, 2009). Phytochelatins can bind Cd directly, reducing its cytoplasmic toxicity. The application of $CdSO_4$ enhances the sulphate concentration and could subsequently render higher Cd tolerance. By using $Cd(NO_3)_2$, more nitrates are introduced into the growth system. Fujita *et al.* (2010) demonstrated that increased N supply stimulates phosphatase activity due to the altered N:P ratio. On top, P starvation is primary due to high N:P ratios and to a lesser extent to low P supply (Duff *et al.*, 1994). Due to their limited availability in the soil, N, P and K (i.e. primary plant macronutrients) are the limiting factors for plant growth. Using $Cd(NO_3)_2$, additional nitrates are supplied to the growth system, potentially resulting in an increased plant growth. Although this effect depends on the ratio of the applied amount of $Cd(NO_3)_2$ to the amount of nitrates present in the growth system, one should apply the same amount of nitrates to the control group using e.g. KNO_3. Considering that the concentration of chlorine in Hoagland solution is only 1.82 µM, the utilization of $CdCl_2$ demands more precaution. All included hydroponic experiments use (a diluted) Hoagland solution. In soil cultivation systems fertigation can be used as well, but the composition is hardly ever specified. Although chlorine is classified as a micronutrient, it is toxic at millimolar range. Using $CdCl_2$, the amount of chlorine is increased dramatically as compared to the controls. Therefore, the effects described using $CdCl_2$ should be interpreted carefully, since they can be the result of the presence of Cd or the relative strong increase in chlorine concentration. Moreover, multiple studies show that the presence of chlorine in the soil results in higher amounts of available Cd in the soil (Prasad, 1995; Smolders an McLaughlin, 1996; Bingham *et al.*, 1984; Doner, 1978) and in an increased Cd uptake in plants (Giordano *et al.*, 1983).

From the 13 studies included in this review, 4 used $CdCl_2$, 6 used $CdSO_4$ and 3 have not specified the chemical form they used (Table 4.2.1). For the articles using $CdSO_4$, we believe there will not be secondary effects since the applied concentration is far below the concentration of SO_4^{2-} in the Hoagland solution. However, $CdCl_2$ was used in very high amounts (10 up to 500 µM) and gave a much higher chlorine concentration than the one initially present in the watering

solution (from ± 20 times up to ± 550 times higher; calculated if watering solution was specified). Thus, data produced by experiments using $CdCl_2$ should be interpreted carefully, since secondary effects due to higher chlorine concentration in Cd-exposed plants vs. controls could be present.

4.2.2.1.5. The applied exposure time

The exposure time ranges from 2 hours to 70 days. Depending on the plants' metabolic speed, a threshold to classify "short" and "long" exposure time is difficult to preconceive. In most time scale studies, the exposure time is kept under or above 3 days. Therefore, 3 days will be the threshold to distinguish between 'short' and 'long' exposure times in this review; all exposure times below or equal to 3 days will be considered 'short' term and all exposure times of more than 3 days will be considered 'long' term.

4.2.2.1.6. The studied tissues and organs

A variety of tissues and organs is used across the studies that focus on the proteome response of plants to Cd. Since proteome patterns depend on the studied tissues and organs, one needs to take this into account when drawing conclusions. The comparison between hydroponics and soil systems (see section 4.2.6) focuses only on leaf and root samples. However, comparison still remains though since major differences are even present in the same organs. For example, Fagioni and Zolla (2009a) described proteome differences studying the effect of Cd in basal and apical spinach leaves. This was confirmed by Jorrín-Novo *et al.* (2009) and on top they state that the 2-DE pattern depends on leaf orientation (North, East, South and West) and the time of the day when leaves are collected.

4.2.2.2. Impediments with regard to proteomic analysis

4.2.2.2.1. The diversity of material and methods peculiar to proteomics: from extraction to differential expression

Focussing on the proteome, one has a huge diversity of extraction protocols, staining methods, equipment and software to choose from. Proteins can be **extracted** in several ways, using e.g. TCA/aceton, extraction buffers or phenol and can be subjected to numerous methods of fractionation. Phenol-based extractions are considered to enhance the extraction of glycoproteins (Ahsan *et*

al., 2009). Furthermore, Cilia *et al.* (2009) demonstrated that each protein extraction method isolates a distinct "extractome". Comparing TCA/acetone, phenol and multi-detergent extraction, they observed that 26.7% of the total spots were differentially extracted by one or two of the three methods. Protein extraction methods are therefore believed to be complementary to one another (Ahsan *et al.*, 2009; Carpentier *et al.*, 2005). Sample **prefractionation** receives increasing attention since it reduces sample complexity and dynamic range, two features that could overwhelm the capacity of all currently available analytical platforms (Jorrín-Novo *et al.*, 2009). Since extraction and purification of proteins is a critical part of proteomics, researchers should be aware of the fact that the efficiency of a proteomic study relies on the quality of the biological sample used (Ahsan *et al.*, 2009). Another challenge is the **protein quantification**. Since all methods have their specific drawbacks, researchers should evaluate which method is most qualified for their experimental design (Weist *et al.*, 2008). However, the reproducibility of the assay is more important since one should always load the same amount of proteins for each sample on the gel. Different techniques are available to **separate proteins**, either gel-based or gel-free, both with their own advantages and disadvantages (for reviews, we refer to Ashan *et al.*, 2009; Wilm, 2009; Miller *et al.*, 2010; Vaudel *et al.*, 2010). **Staining methods** greatly differ in sensitivity, dynamic range, reproducibility and compatibility with identification methods. A great improvement was established by the introduction of fluorescent dyes (e.g. CyDyes), which rendered even higher sensitivity and allowed to analyse multiple samples on the same gel. Moreover, comparison between individual gels was more feasible since an internal standard can be used on each gel. Nevertheless, no staining method provides complete staining of the proteome (van den Broeck *et al.*, 2008). Moreover, **software packages** differ in their analysis resulting in differences in the sets of proteins discovered. Numerous software programs are developed to match gels and protein spots for differential protein analysis, claiming to automate the matching and thereby providing accurate gel analysis. Recently, five commercially available software programs were tested, showing that less than 3% of the total processing time was automated, 1-62% of correct matches were generated automatically and that increasing the number of replicates

decreased the number of automatically correct matches (Clarck and Gutstein, 2008).

Other factors that are almost never reported, but can have a huge impact on proteins, are the **storage conditions** of protein samples and **the thaw –freeze cycles** that protein samples are subjected to (Weist *et al.*, 2010). Therefore it is recommended to work with aliquots of your protein samples to minimize the thaw – freeze cycles, as suggested by Weist *et al.* (2010).

The obtained proteins in a study are just a 'snapshot' of the total proteome. This snapshot greatly depends on the used methods, making it very hard to validate studies using different techniques (Alvarez *et al.*, 2009). Moreover, in 2006, Wilkins *et al.* stated that the enthusiastic application of new, but immature proteomic techniques to significant biological questions, resulted in numerous publications of questionable quality that require further confirmation and/or validation.

4.2.2.2.2. The identification process

A critical step in quantitative proteomics is the robust and accurate protein identification, since they are essential in understanding the dynamic nature of the proteome (Yu *et al.*, 2010). Issues to be considered performing protein identification by mass spectrometry (MS) were outlined in detail by Baldwin in 2003. As for materials and methods used from extraction up to differential expression, a huge diversity is present in the identification process. **Proteolytic digestion** is mostly performed using trypsin and although this technique is well established, pitfalls could occur since negative interactions between the proteolytic enzyme and sample components can occur, potentially leading to sample losses (Clifton *et al.*, 2011). Optimizing this step is important since it could lead to faster and better performance of proteomic analysis. Since high throughput proteomic analysis were available, **database sizes** increased considerably. However, it should be kept in mind that the number of highly confident identified proteins consistently decreases as database size increases (Searle, 2010). As software packages differ in their analysis, **search engines** differ in the way they score identifications, both resulting in differences in the sets of proteins discovered. Despite the complexity of the available software, a 'black box' application of software should be avoided. To start, when normalising

data, caution should be taken since this could also normalise biological differences (Cairns *et al.*, 2008). Moreover, variations caused by bio-informatic analysis have a major impact on the number of correctly identified proteins (Searle, 2010). Using the target-decoy strategy, the false discovery rate (FDR) can be estimated. The quality control of this strategy was recently examined by Vaudel *et al.* (2011). Balgley *et al.* (2007) have demonstrated that, for large data sets, peptide identifications differ depending on the used search algorithms, especially for peptides scoring close to the threshold for rejection or acceptance. Speculation on whether more identifications could be extracted combining multiple search engines, resulted in the creation of algorithms combining multiple search engines, which give a higher number of identifications accompanied by a lower rate of false positives (Yu *et al.*, 2010; Jones *et al.*, 2009; Searle *et al.*, 2008). For example, Scaffold allows you to combine multiple search engines, by converting search engine scores to probabilities of peptide identification using the Peptide Prophet algorithm. Using a new metric called the "combined FDR score", which appears to be a highly effective discriminator for correct/incorrect identifications, Jones *et al.* (2009) reported a higher percentage in identifying proteins than Scaffold. In 2010, the MSENC (Multiple Search Engines, Normalisation and Consensus) algorithm was created by Yu *et al.*, including 6 search engines and a re-scoring engine to search MS/MS spectra against protein and decoy sequences. We recommend combining multiple search engines, since it could enhance identification reproducibility and decrease the FDR, making it easier to compare independent experiments. Jones *et al.*, 2009 showed that the combination of the top two search engines enables you to identify almost as many peptides as using three search engines, indicating that adding additional search engines would have limited benefits. On top, the number of search engines combined, is negatively correlated with sensitivity (Yu *et al.*, 2010). Recently the surplus value of these above-described techniques (referred to as 'multisearch' techniques by Tharakan *et al.* (2010)) is questioned and the use of multipass techniques is suggested (Tharakan *et al.*, 2010). These multipass techniques attempt to correctly assign more of the spectra acquired by MS without substantially increasing running time or false positives by tuning the search space (defined as the total number of comparisons between theoretical and experimental mass ladders). In addition to maximize the number

of identified proteins in a sample, an attempt has been made to easily validate borderline hits of database searches by Thomas and Shevchenko (2008). Another approach recently suggested is the use of customized databases, which are derived from existing databases by applying one or more pre-processing steps. This could lead to better identifications, resulting in the creation of 'Database on Demand' (Reisinger and Martens, 2009). In addition, the pre-processing of MS/MS spectra can increase sensitivity of peptide identification and decrease size file and runtime without compromising specificity. Pre-processing methods must be chosen carefully since their performing capacities depend on the search engines used and since not all methods improve the number of identified peptides (Renard *et al.*, 2009). Filtering strategies for MS/MS identifications can be applied to enhance identification, and can be divided into two groups: (I) pre-filtering strategies which distinguish poor MS/MS spectra prior to database search and (II) post-filtering strategies which verify peptide identification rendered by the search engines. Both strategies, their relative merits and potential drawbacks are reviewed by Salmi *et al.* (2009). Other applications that have been proposed to further increase the sensitivity and specificity of current peptide identification engines are listed in Degroeve *et al.* (2011).

Since different MS/MS spectrum processing algorithms are available, the evaluation of their overall efficiency and usefulness is necessary. This evaluation can be done by quantifying the differences in reproducibility between the different algorithms for peak intensity normalization (Degroeve *et al.*, 2011).

Of the articles included in this review, most reported sufficient information to validate identifications. Sometimes, no information is provided concerning the number of peptides used (five out of 33 articles), the used probability thresholds or protein scores (six out of 33 articles), search parameters (nine out of 33 articles), etc. Twenty-two articles reported on all of the three above mentioned points and four articles reported on none of the abovementioned points. Due to this lack of information, it is often not possible to critically evaluate the identification process.

4.2.2.2.3. Statistics to determine differentially abundant proteins

Out of the 33 articles concerning proteomic response to cadmium exposure in plants, 11 articles did not report the statistical test used to point out the differentially abundant proteins. To be thorough, the corresponding authors were contacted to obtain the statistical information, however upon submitting this meta-analysis, still 8 authors failed to respond (Table 4.2.1). From the 22 articles specifying the statistics, 12 used a parametric univariate test and a multiple comparison FDR correction was only specified in one article (Durand *et al.*, 2010). When more than two conditions are compared, a one way ANOVA is used (six out of 22 articles) and a multiple comparison had to be conducted afterwards, however only three articles specified the multiple comparison test. A two way ANOVA was used to test more than two conditions on two independent variables in three out of 22 articles and in none of these studies a multiple comparison was used afterwards (personal comments of the authors). Only two articles used a non-parametric test on their dataset; the Mann Whitney U test or the Kruskall Wallis test. The ANCOVA test was used in only one article, in addition to a two way ANOVA. Usually, the purpose of ANOVA is to compare means between multiple groups/samples or variables/measurements. In analysis of covariance (ANCOVA), a regression analysis is simultaneously performed to evaluate the relationship between one or more (independent) variables (covariates) with the dependent variable(s). Three articles reported (by replying on email) not to have used any statistics at all.

The aforementioned clearly indicates that parametric tests are mostly used to analyze proteomic datasets (paired t-test, student t-test, ANOVA or two way ANOVA). Only four out of 33 studies used multivariate exploratory analysis or combined both. Using these parametric tests assumes normally distributed observations and homogeneous variances displayed by the different groups. On top, using unpaired parametric tests, the observations need to be independent. As recently stated by Lilley *et al.* (2011), it is well known that several of these assumptions are not met in proteomic datasets. Transformation of the data can leverage the requirements, but sometimes non-parametric tests have to be applied at the expense of the statistical power (See section 4.2.5).

4.2.2.2.4. The applied fold changes

Differential abundance among proteins is usually expressed by fold changes. Fold changes are defined as the ratio of the final value to the initial value. Fold changes higher than 1 might indicate increase in abundance, while fold changes lower than 1 might indicate decrease in abundance. Sometimes fold changes are expressed as a negative value to indicate decrease in abundance. After statistical analysis, lists of differentially abundant proteins are generated, each with their specific fold change. Comparing the included articles, fold changes range from |0.02| up to |36.78|. In proteomic literature, there is no consistency in using a threshold for fold changes. Some authors accept all fold changes, while others only accept e.g. fold changes above 3 or below -3. This inconsistency results in (1) either misleading information due to false positives or (2) in a loss of proteins due to false negatives. A big difference in fold change can be false positive when the variability is high, while a small difference can be significant and false negative when the variability is small and the number of replicates high. Only an objective and appropriate statistical test can judge about relevant fold changes. For an ideal meta-analysis, all authors should be encouraged to release their raw data so that different statistical tests can be re-applied to create an objective description of the data.

Analyzing 169 articles, Petrak *et al.* (2008) have shown that some proteins seem to predominate regardless of the experiment, species or tissues analyzed. This might confirm the existence of a general stress response (Petrak *et al.*, 2008). However it could also be explained by the fact that proteins with high abundance, which are easy to identify, are mostly observed at the expense of the lower abundant ones. Keeping this in mind, researchers should be critical about the identified protein list generated by their experiment. The meta-analysis of Petrak *et al.* (2008) focused on human and rodent samples, however we believe it would be valuable to generate such a list for plant tissue as well.

4.2.3. Consensus in proteomic research... An illusion or plausible?

Keeping in mind that the final result can only be as good as the starting material, protein extraction is one of the most critical steps. It is difficult to point out a general good performing extraction protocol since this is highly tissue dependent. Therefore, we believe researchers should first focus on selecting the

most appropriate extraction protocol for their samples. This should, ideally, render the highest number of distinct protein spots, be highly reproducible, contain as less contaminants as possible and minimize artificial protein degradation and modification. In the subsequent steps of the proteomic workflow, the available techniques, protocols, materials, software packages, algorithms, etc. are even larger, making the generated protein list dependent on the workflow chosen by the researcher. Proposing a general workflow is unrealistic since no workflow is able to generate the full proteome. As a result, analyzing proteomic literature, data provided on a specific topic could only be interpreted as complementary to each other. 'Comparing' proteomic studies between laboratories is almost impossible, since each study only renders a specific sub-proteome. As stated by Lilley *et al.* (2011), the use of homemade standards may satisfy the need for an internal standardization, but does not lend itself to cross-laboratory standardization.

Despite the fact that no consensus can be reached for the proteomic workflow, we believe general guidelines are indispensable. Although frequently used in the past decades, proteomics is still in its infancy and at a highly evolving stage. Tremendous research is focussing on optimizing the proteomic workflow at almost every level. This has to be encouraged in order to get a better reproducible, high-throughput workflow. On the other hand, up to date so many studies are available suggesting improvements that it is impossible, as a non-specialist, to determine which method is the most accurate to achieve this goal. Various research groups are developing tools for proteomic research, analysis, database development, etc. For the sake of the proteomic community, these research groups should bundle their forces instead of competing with each other. Since the workflow of proteomics encounters so many protocols, equipments, software packages, search algorithms, ... it is impossible to understand each step in depth. In addition, Agrawal *et al.* (2011) stated that we lack a suitable globally recognized platform along with centralized and user-friendly database and that media coverage and information dissemination should be enhanced. Therefore, we strongly believe that the field of proteomics needs to assign a group of specialists at every level of the proteomic workflow. Their aim should be to follow up and critically evaluate all suggested improvements and to generate a guideline for methods to be used at each stage of the

proteomic workflow. These guidelines should be regularly updated, since research to improve proteomics is still largely ongoing and should be easily accessible for all researchers. Only in this way it will be possible to render good valid proteomic results, since it is impossible for a researcher to be specialised at each level of the proteomic workflow. In order to concede to the dissemination of proteomic information, the INPPO (International Plant Proteomics Organization) aims at establishing complete plant proteomes by properly organizing, preserving and disseminating collected information on plant proteomics. The INPPO is a good initiative towards centralizing plant proteomic information, however their contribution to a general proteomic guidelines, as stated above, is to be reviewed later on. Since the success of this project depends on researchers input, we highly encourage plant proteomic researchers to join the INPPO project.

4.2.4. Validation and good reporting practice for protein identification

Mass spectrometry is used at large scale in proteomic research. Since a broad range of researchers are not specialised in MS, the major limitations of the system that critically affect the reliability and significance of the identifications are substantially less understood and sometimes less considered (Baldwin, 2003). Since proteomics is inextricably bound up with MS, the quality of the outcome of a proteomic experiment is strongly dependent on the quality of the mass spectra underpinning protein identifications (and quantifications) as returned by a search engine from a primary sequence database. Databases should be under critical evaluation since proteome coverage, and thus protein identification, strongly depends on size and quality of reference protein databases by which obtained MS and MS/MS data are compared (Schneider and Riedel, 2010).

It is challenging to establish criteria for identification of proteins by mass spectrometry. Some general precautions should be taken into account. Each automatically-generated identification needs to be manually verified, even if a preset cross-correlative or statistical threshold is met and/or a refined expert system is in use such as Amass (Sun *et al.*, 2004), Peptizer (Helsens *et al.*, 2008) or Scaffold™. This in turn requires an understanding of peptide spectra. Hereby, two criteria generally apply: the fragment ions should be clearly above

baseline noise and the spectrum should have continuous b or y ion matches (Link *et al.*, 1999). Dependent on the occurrence of specific amino acids in the peptide such as proline, acidic and/or basic amino acids, additional criteria may apply. For these and other peculiarities about mass spectral interpretation the (novice) reader might be referred to a 'tutorial' paper (Jonscher, 2004). Furthermore, protein identifications relying on one peptide only ('one-hit wonders') need special care and above all, mass spectral documentation. The same applies to peptide modifications that possibly are of co-/post-translational origin. Parameter settings for identification strategies are typically far less rigorously controlled (Vaudel *et al.*, 2011). Optimal search parameters can be found in Vaudel *et al.* (2011) and the peptide mass tolerance and modification parameters can be subjected to further optimization as described therein. In current practice, a proteomic study results on many occasions in the discovery of a (set of) protein(s) enabling the differentiation between at least two conditions. Optimally, one should validate the mass spectral-driven outcome of such an experiment by an orthogonal approach such as western blotting or ELISA. The latter allows for a high throughput evaluation to validate many different samples, a characteristic that still is lacking in nowadays proteomics since the LC step is time consuming.

Because in proteomics experimental designs and analytical techniques are becoming increasingly complex, good reporting practice is of utmost importance. Criticizing the poor experimental setup, the poor statistical analysis, the poor information that is reported in articles, the non-systematic standard for protein identification, etc., Wilkins *et al.* (2006) stated that guidelines are needed for further proteomic analysis. Since then, consensus guidelines such as the Paris guidelines (imposed by the journal 'Molecular and Cellular Proteomics'; http://www.mcponline.org/site/misc/ParisReport_Final.xhtml) or the HUPO initiative 'Minimum Information About Proteomics Experiments' ('MIAPE'; http://www.psidev.info/miape/) or the guidelines of the PROTEOMICS journal (Wilkins *et al.*, 2006; http://hal.archives-ouvertes.fr/docs/00/37/33/22/PDF/guidelines.pdf), have been established. The HUPO initiative aims to develop a set of MIAPE modules specifying - besides a controlled vocabulary - the suitable level of details required when reporting proteomic investigations. MIAPE-compliant reporting will strongly favor the

standardized collection, integration, storage and dissemination of proteomic data (Taylor *et al.*, 2007) coming from experiments using techniques such as gel electrophoresis (Gibson *et al.*, 2008), column chromatography (Jones *et al.*, 2010), mass spectrometry (Taylor *et al.*, 2008) as well as proteomic informatics (Hoogland *et al.*, 2010; Vizcaíno *et al.*, 2009). Finally, user submission of proteomic mass spectral data in a public repository such as PRIDE[11] forms also an essential part of good reporting practice.

4.2.5. Guidelines for statistical analysis of differentially abundant proteins

After separation through two-dimensional gel electrophoresis (2-DE) or LC several hundreds of individual protein or peptide abundances can be quantified. Both, a good experimental setup and a valid statistical approach are essential to get insight into the data and to draw correct conclusions (Carpentier *et al.*, 2007). High throughput proteomic experiments yield complex and large datasets with a huge disproportion between the hundreds of variables and the restricted number of replicates. Though, most commonly used statistical tests have been designed to cope with a high number of replicates and a restricted number of variables. There is some inconsistency in the proteomic community related to the use of statistics. Proteomic data are currently analyzed by a variety of approaches. Two approaches of data analysis can be distinguished: exploratory data analysis and confirmatory data analysis. Currently, most proteomic data are analyzed with the emphasis on confirmatory analysis (Karp and Lilley, 2007). This was also the case with the studies concerning proteomic response to cadmium (29 out of 33 reported studies use only confirmatory data analysis).

Univariate confirmatory methods examine the individual proteins/peptides one by one, considering them as independent measurements. Univariate methods start from the null hypothesis that there is no difference between the experimental populations. Parametric univariate statistical tests are very powerful but the data must respect the restrictive assumptions (continuous and normally distributed data, homogeneity of variance and independent samples) and the assumptions must be tested. Transformation of data (e.g. log function, arcsine, square root) are frequently used to improve the distribution

characteristics (normality and homogeneity of variance) (Gustafsson *et al.*, 2004). Parametric models like the Student's T-test and ANOVA (analysis of variance) start from the observed sampling and assume that the observed sample mean and variance approximate the real population mean and variance, and that the variances of the experimental populations are equal. Based on the observed mean and variance, the populations are considered normally distributed and a model is made. If the test statistic is large enough, the null hypothesis is rejected. Given the labor and cost involved in the 2-DE analysis, the number of replicates is often restricted and ranges usually between 3 and 6. Although some empirical evidence illustrates that slight deviations in meeting the assumptions underlying parametric tests may not have radical effects on the obtained probability levels, there is no general agreement as to what is a 'slight' deviation (Siegel and Castellan, 1988). The low sample size is also a big bottle neck to check the assumptions of the parametric tests (Neter *et al.*, 1996). Transformation of the data can leverage the requirements, but sometimes non-parametric tests have to be applied at the expense of the statistical power. Non-parametric tests do not assume any distribution. The assumptions are independent and continuous ordinal data. A non-parametric test determines whether or not the experimental groups come from the same distribution. Therefore, the data points in each experimental group are sorted in ascending order and an empirical distribution function is calculated without any assumption of distribution or variance. Useful non-parametric tests are the Kolmogorov-Smirnov test, the Mann Whitney U test or the Kruskall Wallis test.

However, univariate statistical tests have not been designed to analyze complex datasets containing multiple correlated variables. Proteomic datasets generally contain hundreds of different proteins/peptides that are correlated. Peptides are derived from proteins and proteins fit within the larger entity of networks and interact with each other. Moreover, testing hundreds of variables one by one and reporting them with an acceptance of a certain risk of false positives (α), enhances the chance of reporting false positive cases (multiple testing issue) and need to be corrected. To retain a general error rate α in an analysis involving more than one comparison, the error rate for each comparison must be more stringent than the original individual α. The Bonferroni correction is the most simple used approach for multiple comparisons and states that if each test

is performed to have type I error rate ɑ/n, the total error rate will not exceed ɑ, where n is the number of tests. However in a proteomic experiment n reaches easily > 1000 and the actual general error rate is much less than the original level ɑ. This threshold is generally too stringent. Commercial proteomic software generally are aware of this issue and imply a less stringent algorithm referred to as False Discovery Rate (FDR).

A valuable alternative for parametric and non-parametric confirmatory tests are the exploratory analysis techniques. The field of multivariate analysis consists of those techniques that consider multiple related random variables as a single entity and attempts to produce an overall result taking the relationship among the variables into account (Jackson, 2003). In contrast to a univariate approach, it displays the interrelationships between the large number of variables and is able to correlate multiple peptides/proteins to a specific experimental group. Principal Component Analysis (PCA) is one of the multivariate possibilities to perform explorative data analysis that starts to be commonly used in proteomic experiments. The most logical modus operandi is to consider the different biological replicate samples of the experimental groups as observations (score plot). As such trends and grouping of samples might be detected and allows studying which proteins are important to explain trends and grouping in the data through a loading plot. A principal axis transformation transforms the correlated variables (proteins/peptides) into new uncorrelated variables. A principal component is a linear combination calculated from the existing variables (proteins/peptides). The relation between the original variables (proteins/peptides) and the principal components is displayed in the loading plot. This means that if a protein/peptide has a high loading score for a specific principal component, that this protein/peptide explains an important part of the sample variance. Multivariate statistics have an additional value by being capable to differentiate the different experimental groups in terms of correlated expression rather than absolute expression. Uni- and multivariate approaches are complementary. Performing the analysis only on the significant proteins from univariate analysis might disregard useful information.

4.2.6. Cadmium response – time and culture comparison

Focussing on mature plants, the primary aim of this meta-analysis was to compare the general stress response of plants to Cd between (1) long and short-term exposures and (2) hydroponic and soil cultivation systems. To make the comparison more straightforward, we focused on (1) studies applying only Cd and comparing this to control conditions, (2) studies using leaf and/or root tissues and (3) studies working with hydroponic and/or soil cultivation systems. Mixed contaminations, cell suspension cultures, seeds, algae, etc. and studies on MS medium, Petri-dish cultivation, ... are excluded from further discussion (Table 4.2.1). After this selection, 13 articles remained and are included in this meta-analysis. Throughout the 13 studies included in this comparison, a huge diversity was present in experimental setup, materials, methods and data analysis (Table 4.2.1). Since each combination of methods leads to a specific sub proteome, we believe the results obtained from the different 'workflows' are complementary to each other. Therefore, no distinction between techniques, protocols, used instruments and software, etc. was made in this meta-analysis. However, a selection was made based on statistical analysis of differentially abundant proteins, where articles not reporting or performing statistics are excluded from the meta-analysis.

Since the significance of fold changes depends on among others, the amount of biological and technical replicates, all proteins reported in the articles as significantly differentially abundant, were included in this meta-analysis, regardless of their fold change. One exception was made: the protein lists reported by Kieffer *et al.* (2008, 2009a, 2009b) were generated by a two way ANOVA with control/exposed and sample dates as independent variables. The analysis was carried out in Decyder, which does not allow for multiple comparisons for two way ANOVA. Therefore the authors used a threshold of 1.5 to distinguish at which time points the proteins are significantly differentially abundant (P. Kieffer, personal comments). Only the proteins that were considered significantly differentially abundant by the authors (those with a fold change > |1.5|) were used in this meta-analysis.

The reported proteins in these 13 articles are divided into four **conditional groups** (Table 4.2.3): (1) hydroponic cultivation system – short term exposure, (2) hydroponic cultivation system – long term exposure, (3) soil cultivation

system – short term exposure and (4) soil cultivation system – long term exposure. Short term exposure includes studies with an exposure time up to 3 days, while long term exposure refers to studies with an exposure time longer than 3 days (see section 4.2.2.1.5 for discussion). Each group is subdivided in leaf and root proteins. In some conditional groups only a few proteins are present, while in others over 450 proteins are present (Table 4.2.4). In Table 4.2.5, a comparison between the cultivation method, exposure time and sample type is made for the amount of proteins reported and the amount of articles involved. Upon comparison, most articles focus on hydroponic cultivation system, long term exposure and leaf samples.

Table 4.2.3: Distribution of articles included in the meta-analysis among different conditional groups.

	Hydroponic cultivation system		Soil cultivation system	
	Leaf	Root	Leaf	Root
Short	Kieffer *et al.* 2008	Roth *et al.* 2006	-	Alvarez *et al.* 2009
	Kieffer *et al.* 2009b	Kieffer *et al.* 2009b		
	Zhoa 2011			
Long	Kieffer *et al.* 2008	Rodriguez-Celma *et al.* 2010	Durand *et al.* 2010	Repetto *et al.* 2003
	Kieffer *et al.* 2009a	Kieffer *et al.* 2009b		Aloui *et al.* 2009
	Kieffer *et al.* 2009b			
	Fagioni and Zolla 2009a			
	Semane *et al.* 2010			
	Visioli *et al.* 2010			

Table 4.2.4: Number of differentially abundant proteins grouped per condition.

			UP	DOWN	Total proteins per class	Percentage of total proteins (of all classes)
Short	Hydroponic cultivation system	Leaf	14	18		
		Root	20	1	53	6.6
	Soil cultivation system	Leaf	0	0		
		Root	76	86	162	20.3
Long	Hydroponic cultivation system	Leaf	201	143		
		Root	44	89	477	59.7
	Soil cultivation system	Leaf	28	58		
		Root	17	4	107	13.4

Table 4.2.5: Number of proteins grouped per cultivation system, exposure time and studied tissue.

	Nr of proteins	Nr of articles	Percentage of total proteins
Hydroponic cultivation system	530	9	66.3
Soil cultivation system	269	4	33.7
Short term exposure	215	5	26.9
Long term exposure	584	10	73.1
Leaf samples	462	8	57.8
Root samples	337	6	42.2

A protein list containing the NCBI gi accession number or the EST accession number was generated for each subgroup. Subsequently, all numbers were submitted to “Batch Entrez” (http://www.ncbi.nlm.nih.gov/sites/batchentrez) to

subtract fasta files. Next, all fasta files were analyzed by Blast2Go (version 2.4.8; http://www.blast2go.org/start_blast2go) to get an annotation. During blasting, BLASTp was performed on protein sequences and BLASTx was performed on EST sequences. In total, 14 proteins had to be left out since no gi number could be retrieved for them; for two proteins no accession number was given by the authors and for 12 proteins the given accession number was no longer present in the corresponding database. The confirmed proteins are listed in Table S1 (Supplementary data) and all proteins were classified into **functional groups**. Of a total of 799 reported proteins, 260 proteins belonged to the carbohydrate metabolism, 166 were involved in stress metabolism, 164 in protein metabolism, 68 proteins belonged to the energy metabolism, 31 proteins were involved in nucleotide metabolism, 18 proteins in transport mechanisms and 14 proteins were involved in cell structure, maintenance, growth or death. Additionally, 68 proteins were grouped as "others" and 10 proteins were reported as hypothetical or unknown protein. The stress metabolism is subdivided into two subgroups: (1) "Detoxification"; comprising proteins that are involved in metabolism of toxic compounds such as ROS and (2) "Defence"; comprising proteins that are reported to be involved in response to stress such as PR proteins. The energy metabolism comprises light reactions of photosynthesis and mitochondrial electron transport chain. The carbohydrate metabolism is divided into (1) "CO_2-fixation"; comprising carbon reactions of photosynthesis, (2) "Catabolism"; comprising glycolysis/gluconeogenesis, TCA cycle, pyruvate metabolism and pentose phosphate pathway and (3) "Metabolism of C-based molecules"; comprising all other carbohydrate metabolism subgroups, as listed in the KEGG PATHWAY database (http://www.genome.jp/kegg/pathway.html). These groups were chosen to get a better insight of the energy flow during Cd exposure.

The hereafter given data are based upon the protein list in Table S1 (supplementary data). After functional grouping, Table 4.2.6 was extracted, giving the absolute number of proteins of each conditional subgroup, grouped per function as well as their corresponding percentage. Percentages represent the amount of up- or down-regulated proteins that are involved in a particular function out of the total proteins present in each conditional subgroup.

Table 4.2.6: Total number of proteins in each conditional group per functional group. 'All proteins' represent the sum of all differentially abundant proteins per function from all conditional groups. Proteins of all conditions were considered together. Sum of subgroups is represented in bold. (N.S.SG = no specified subgroup). ABS represents the absolute number of proteins, percentage (Italic) is calculated to the total differentially abundant (in- and decreased) proteins per conditional subgroup.

FUNCTIONAL GROUP	All proteins		Hydroponic cultivation system															
			SHORT								LONG							
			LEAF				ROOT				LEAF				ROOT			
			UP		DOWN		UP		DOWN		UP		DOWN		UP		DOWN	
	ABS	%	ABS	%	ABS	%	ABS	%	ABS	%	ABS	%	ABS	%	ABS	%	ABS	%
Carbohydrate metabolism	**260**	***32.5***	**1**	***3.1***	**13**	***40.6***	**4**	***19.0***	**0**	***0.0***	**63**	***18.3***	**56**	***16.3***	**10**	***7.5***	**51**	***38.3***
Carbohydrate metabolism - N.S.SG	7	*0.9*	0	*0.0*	1	*3.1*	1	*4.8*	0	*0.0*	2	*0.6*	2	*0.6*	0	*0.0*	0	*0.0*
Carbohydrate metabolism - CO2 fixation	95	*11.9*	0	*0.0*	12	*37.5*	0	*0.0*	0	*0.0*	10	*2.9*	46	*13.4*	0	*0.0*	0	*0.0*
Carbohydrate metabolism - Metabolism of C-based molecules	21	*2.6*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	2	*0.6*	0	*0.0*	3	*2.3*	4	*3.0*
Carbohydrate metabolism - Catabolism	137		1	*3.1*	0	*0.0*	3	*14.3*	0	*0.0*	49	*14.2*	8	*2.3*	7	*5.3*	47	*35.3*
Cell	**14**	***1.8***	**0**	***0.0***	**1**	***3.1***	**1**	***4.8***	**0**	***0.0***	**3**	***0.9***	**2**	***0.6***	**1**	***0.8***	**0**	***0.0***
Cell - Death	2	*0.3*	0	*0.0*	0	*0.0*	1	*4.8*	0	*0.0*	1	*0.3*	0	*0.0*	0	*0.0*	0	*0.0*
Cell - Structure, growth and maintenance	12	*1.5*	0	*0.0*	1	*3.1*	0	*0.0*	0	*0.0*	2	*0.6*	2	*0.6*	1	*0.8*	0	*0.0*
Energy metabolism	**68**	***8.5***	**1**	***3.1***	**0**	***0.0***	**0**	***0.0***	**0**	***0.0***	**18**	***5.2***	**23**	***6.7***	**1**	***0.8***	**2**	***1.5***
Energy metabolism - N.S.SG	1	*0.1*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	1	*0.8*
Energy metabolism - Production	67	*8.4*	1	*3.1*	0	*0.0*	0	*0.0*	0	*0.0*	18	*5.2*	23	*6.7*	1	*0.8*	1	*0.8*
Nucleotide metabolism	**31**	***3.9***	**0**	***0.0***	**0**	***0.0***	**2**	***9.5***	**0**	***0.0***	**1**	***0.3***	**3**	***0.9***	**0**	***0.0***	**2**	***1.5***
Nucleotide metabolism - N.S.SG	2	*0.3*	0	*0.0*	0	*0.0*	2	*9.5*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*
Nucleotide metabolism - Synthesis/Degradation	3	*0.4*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*
Nucleotide metabolism - Transcription/Translation	26	*3.3*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	1	*0.3*	3	*0.9*	0	*0.0*	2	*1.5*
Protein metabolism	**164**	***20.5***	**5**	***15.6***	**1**	***3.1***	**3**	***14.3***	**0**	***0.0***	**43**	***12.5***	**24**	***7.0***	**13**	***9.8***	**16**	***12.0***
Protein metabolism - Synthesis	87	*10.9*	3	*9.4*	1	*3.1*	2	*9.5*	0	*0.0*	22	*6.4*	5	*1.5*	1	*0.8*	10	*7.5*
Protein metabolism - Folding	44	*5.5*	1	*3.1*	0	*0.0*	0	*0.0*	0	*0.0*	6	*1.7*	17	*4.9*	8	*6.0*	2	*1.5*
Protein metabolism - Degradation	33	*4.1*	1	*3.1*	0	*0.0*	1	*4.8*	0	*0.0*	15	*4.4*	2	*0.6*	4	*3.0*	4	*3.0*
Stress metabolism	**166**	***20.8***	**4**	***12.5***	**0**	***0.0***	**8**	***38.1***	**1**	***4.8***	**49**	***14.2***	**22**	***6.4***	**15**	***11.3***	**14**	***10.5***
Stress metabolism - N.S.SG	6	*0.8*	0	*0.0*	0	*0.0*	1	*4.8*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*
Stress metabolism - Defense	67	*8.4*	1	*3.1*	0	*0.0*	2	*9.5*	1	*4.8*	23	*6.7*	4	*1.2*	6	*4.5*	4	*3.0*
Stress metabolism - Detoxification	93	*11.6*	3	*9.4*	0	*0.0*	5	*23.8*	0	*0.0*	26	*7.6*	18	*5.2*	9	*6.8*	10	*7.5*
Transport	**18**	***2.3***	**0**	***0.0***	**0**	***0.0***	**0**	***0.0***	**0**	***0.0***	**2**	***0.6***	**0**	***0.0***	**0**	***0.0***	**0**	***0.0***
Unknown protein/hypothetical protein	**10**	***1.3***	**0**	***0.0***	**0**	***0.0***	**2**	***9.5***	**0**	***0.0***	**1**	***0.3***	**3**	***0.9***	**0**	***0.0***	**0**	***0.0***
Others	**68**	***8.5***	**3**	***9.4***	**3**	***9.4***	**0**	***0.0***	**0**	***0.0***	**21**	***6.1***	**10**	***2.9***	**4**	***3.0***	**4**	***3.0***
Total per column	799	*100.0*	14	*43.8*	18	*56.3*	20	*95.2*	1	*4.8*	201	*58.4*	143	*41.6*	44	*33.1*	89	*66.9*
Total proteins per condition (up and down regulated)			32				21				344				133			
Number of articles per condition			3				2				6				2			

Table 4.2.6: Total number of proteins in each conditional group per functional group. 'All proteins' represent the sum of all differentially abundant proteins per function from all conditional groups. Proteins of all conditions were considered together. Sum of subgroups is represented in bold. (N.S.SG = no specified subgroup). ABS represents the absolute number of proteins, percentage (Italic) is calculated to the total differentially abundant (in- and decreased) proteins per conditional subgroup (Continued).

FUNCTIONAL GROUP	All proteins		Soil cultivation system														
			SHORT						LONG								
			LEAF		ROOT				LEAF				ROOT				
			UP	DOWN	UP		DOWN		UP		DOWN		UP		DOWN		
	ABS	%			ABS	%	ABS	%	ABS	%	ABS	%	ABS	%	ABS	%	
Carbohydrate metabolism	**260**	***32.5***	No reported proteins	No reported proteins	**10**	***6.2***	**10**	***6.2***	**14**	***16.3***	**26**	***30.2***	**3**	***14.3***	**1**	***4.8***	
Carbohydrate metabolism - N.S.SG	7	*0.9*			0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	1	*4.8*	0	*0.0*	
Carbohydrate metabolism - CO2 fixation	95	*11.9*			0	*0.0*	0	*0.0*	7	*8.1*	20	*23.3*	0	*0.0*	0	*0.0*	
Carbohydrate metabolism - Metabolism of C-based molecules	21	*2.6*			6	*3.7*	1	*0.6*	2	*2.3*	1	*1.2*	2	*9.5*	0	*0.0*	
Carbohydrate metabolism - Catabolism	137				4	*2.5*	9	*5.6*	5	*5.8*	5	*5.8*	0	*0.0*	1	*4.8*	
Cell	**14**	***1.8***			**2**	***1.2***	**1**	***0.6***	**1**	***1.2***	**2**	***2.3***	**0**	***0.0***	**0**	***0.0***	
Cell - Death	2	*0.3*			0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	
Cell - Structure, growth and maintenance	12	*1.5*			2	*1.2*	1	*0.6*	1	*1.2*	2	*2.3*	0	*0.0*	0	*0.0*	
Energy metabolism	**68**	***8.5***			**2**	***1.2***	**0**	***0.0***	**3**	***3.5***	**18**	***20.9***	**0**	***0.0***	**0**	***0.0***	
Energy metabolism - N.S.SG	1	*0.1*			0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	
Energy metabolism - Production	67	*8.4*			2	*1.2*	0	*0.0*	3	*3.5*	18	*20.9*	0	*0.0*	0	*0.0*	
Nucleotide metabolism	**31**	***3.9***			**3**	***1.9***	**20**	***12.3***	**0**	***0.0***	**0**	***0.0***	**0**	***0.0***	**0**	***0.0***	
Nucleotide metabolism - N.S.SG	2	*0.3*			0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	
Nucleotide metabolism - Synthesis/Degradation	3	*0.4*			3	*1.9*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	
Nucleotide metabolism - Transcription/Translation	26	*3.3*			0	*0.0*	20	*12.3*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	
Protein metabolism	**164**	***20.5***			**9**	***5.6***	**37**	***22.8***	**3**	***3.5***	**8**	***9.3***	**0**	***0.0***	**2**	***9.5***	
Protein metabolism - Synthesis	87	*10.9*			8	*4.9*	31	*19.1*	1	*1.2*	2	*2.3*	0	*0.0*	1	*4.8*	
Protein metabolism - Folding	44	*5.5*			0	*0.0*	4	*2.5*	2	*2.3*	4	*4.7*	0	*0.0*	0	*0.0*	
Protein metabolism - Degradation	33	*4.1*			1	*0.6*	2	*1.2*	0	*0.0*	2	*2.3*	0	*0.0*	1	*4.8*	
Stress metabolism	**166**	***20.8***			**28**	***17.3***	**10**	***6.2***	**4**	***4.7***	**2**	***2.3***	**9**	***42.9***	**0**	***0.0***	
Stress metabolism - N.S.SG	6	*0.8*			2	*1.2*	3	*1.9*	0	*0.0*	0	*0.0*	0	*0.0*	0	*0.0*	
Stress metabolism - Defence	67	*8.4*			16	*9.9*	3	*1.9*	1	*1.2*	0	*0.0*	6	*28.6*	0	*0.0*	
Stress metabolism - Detoxification	93	*11.6*			10	*6.2*	4	*2.5*	3	*3.5*	2	*2.3*	3	*14.3*	0	*0.0*	
Transport	**18**	***2.3***			**11**	***6.8***	**2**	***1.2***	**1**	***1.2***	**0**	***0.0***	**2**	***9.5***	**0**	***0.0***	
Unknown protein/hypothetical protein	**10**	***1.3***			**3**	***1.9***	**1**	***0.6***	**0**	***0.0***	**0**	***0.0***	**0**	***0.0***	**0**	***0.0***	
Others	**68**	***8.5***			**8**	***4.9***	**5**	***3.1***	**2**	***2.3***	**2**	***2.3***	**3**	***14.3***	**1**	***4.8***	
Total per column	799	*100.0*			76	*46.9*	86	*53.1*	28	*32.6*	58	*67.4*	17	*81.0*	4	*19.0*	
Total proteins per condition (up and down regulated)					162				86				21				
Number of articles per condition			-		1				1				2				

4.2.6.1. Time comparison

4.2.6.1.1. Hydroponic cultivation system – short term versus long term Cd exposure

When comparing differentially abundant proteins in short versus long term Cd exposure in hydroponic cultivation systems, it immediately stands out that a much higher number of proteins are affected upon long term exposure. This confirms the observation of Kieffer *et al.* (2008) where only 3 proteins differed in abundance after 3 days, 26 after 7 days and 118 after 14 days in their large time scale study. In addition, more leaf proteins were affected than root proteins, independent of the exposure period, however roots are in direct contact with the contaminant. In the next sections we will take a closer look at proteomic changes in leaves and roots.

4.2.6.1.1.1. Leaf proteome

Only three articles focused on the proteome differences in leaves of plants grown hydroponically during short term Cd exposure (Table 4.2.3). Therein, only 32 proteins were described to be differentially abundant, of which 14 proteins were up-regulated and 18 down-regulated. The "high" number of differentially abundant proteins was mainly the result of data reported by Zhoa *et al.* (2011). This could be the result of a specific Cd response in the hyperaccumulator *Phytolacca americana* L., which is considered to accumulate Cd in a significant higher amount than defined for Cd hyperaccumulators or could be due to the – in hydroponic terms – highly applied Cd concentration (44.8 ppm). The large time scale studies of Kieffer *et al.* (2008, 2009b), only reported 6 proteins to differ in abundance in leaves when applying a short term Cd exposure. The low number of differentially abundant proteins could be due to the 'delay effect', meaning that the exposure time was too short to induce significant proteome changes in leaves. This idea was confirmed by the clustering analysis of Kieffer *et al.* (2008) where gels from 3-days-exposed Cd plants cluster together with the control gels. In contrast to the low number of differentially abundant proteins after short term exposure, long term Cd exposure induces drastic proteome changes in leaves of hydroponically grown plants. In total, 344 proteins differed in abundance from which 201 were up-regulated and 143 were down-regulated. This difference is to be expected since the Cd content linearly

increases in time and hence stress exposure happens gradually in leaves (Kieffer *et al.*, 2009b). With 6 articles focusing on differentially abundant leaf proteins of plants grown hydroponically with a long term Cd exposure (Table 4.2.3), this group appeared to be the most studied and comprises the most proteins (Table 4.2.4).

Functional grouping showed that, the carbohydrate metabolism was the most affected (43.7%) after short term exposure, followed by the protein metabolism (18.7%) and stress metabolism (12.5%). After long term exposure the carbohydrate metabolism was also primarily affected (34.6%), followed by the stress metabolism (20.6%) and the protein metabolism (19.5%).

Focusing on carbohydrate metabolism, down-regulation of CO_2-fixation was the main target with 37.5% of the total differentially abundant leaf proteins after short term exposure. Thereby, data represented in this meta-analysis confirmed the general suppression of carbon fixation in leaves of Cd-exposed plants, as stated by several authors (Hajduch *et al.*, 2001; Kieffer *et al.*, 2008; 2009a; Fagioni and Zolla, 2009a; Semane *et al.*, 2010). Upon short term exposure, energy production was almost not affected; only one chloroplastic ATP synthase has been reported to be up-regulated. Upon long term exposure, up-regulated proteins of the carbohydrate metabolism were mainly involved in catabolism, more specifically related to glycolysis and the TCA cycle. The up-regulation of catabolism can be seen as a compensation to the reduced photosynthesis as indicated by the high down-regulation of proteins involved in CO_2-fixation (13.4%). Slowing down carbon fixation creates the need to remobilize energetic storage (Kieffer *et al.*, 2008) and subsequently the precursors for catabolism probably come from the remobilization of stored carbon. As an indication for this statement phosphoglucomutase, which is involved in starch production and mobilization, was up-regulated. Interestingly most of the up-regulated proteins involved in catabolism produce NADH, reducing power that is essential to counterbalance the negative effect of Cd on the ROS balance (Cuypers *et al.*, 2011; Filipic, 2012; Keunen *et al.*, 2012). Among the up-regulated energy production proteins, mitochondrial ATP synthase can be linked to the up-regulation of the TCA cycle. Down-regulated energy proteins are mostly involved in chloroplast electron transport and ATP synthesis. Together with a high down-regulation of proteins involved in CO_2-fixation (13.4%), this clearly indicates a

metabolic shift towards less carbon fixation and a higher respiration. This metabolic shift has been reported several times as a response to Cd exposure (Kieffer *et al.*, 2008; 2009a; Semane *et al.*, 2010). Kieffer *et al.* (2008) linked down-regulation of proteins involved in the light phase of photosynthesis (here classified as "*energy – production*") to the suppression of carbon fixation since the down-regulation of carbon fixation must be counterbalanced to avoid photo-oxidative damage. Finally, it should be mentioned that discrepancies in differentially abundant proteins (Table S1) present between Kieffer *et al.* (2008, 2009a and 2009b) and Fagioni and Zolla (2009a), mainly concerning carbohydrate metabolism, could be a result of the difference in plant species or the used Cd concentration (Table 4.2.1).

Focusing on stress metabolism, proteins in detoxification, such as glutathione-S-transferases and antioxidant enzymes, were more affected than those involved in stress defence, such as pathogenesis related proteins and Kunitz trypsine inhibitors, independent of the exposure period. No down-regulated proteins were reported after short term exposure. Stress metabolism is higher affected upon long term exposure (20.6%) than short term exposure (12.5%). This is in line with the high up-regulation of catabolism after long term exposure that, as described above, is necessary to produce sufficient reducing power. On top, several proteins involved in stress detoxification were reported to be down-regulated upon long term exposure. These down-regulated proteins might be involved in creating a cellular condition of oxidative stress and ROS overproduction that results in the activation of general stress response proteins (Kieffer *et al.*, 2008).

Protein metabolism was more or less similarly affected independent of the exposure period; it comprises 18.7% and 19.5% of all differentially abundant proteins in short term and long term exposures respectively. Protein synthesis was mostly affected, whereas folding and degradation were less affected as compared to protein synthesis. The effect on protein synthesis can be seen as a mechanism to respond accurately to specific stresses (Semane *et al.*, 2010). After long term Cd exposure, up-regulation of protein synthesis mainly involved proteins of glutamine and cysteine synthesis; both amino acids are involved in the GSH synthesis pathway (Sarry *et al.*, 2006; Kieffer *et al.*, 2008; 2009a; Jozefczak *et al.*, 2012). As GSH is a key metabolite in Cd chelation as well as in

the antioxidative mechanism (Jozefczak *et al.*, 2012; Seth *et al.*, 2012), this coincides with the stress metabolism that is clearly activated upon long term Cd exposure.

4.2.6.1.1.2. Root proteome

Although long term Cd exposure on hydroponically grown plants seemed the most popular exposure in studies focusing on leaf proteome (6 studies; 344 differentially abundant proteins), only two articles focused on root proteins under the same conditions (Table 4.2.3). Of the differentially abundant root proteins, 44 were up-regulated and 89 were down-regulated, pointing towards a higher decrease of protein spots as described by Rodríguez-Celma *et al.*, 2010. Comparing the total differentially abundant root proteins revealed the same pattern as described for leaves; long term Cd exposure induced drastic proteome changes, whereas short term Cd exposure only induced moderate changes. This was somewhat unexpected since stress exposure in roots occurs immediately upon the beginning of the exposure (Kieffer *et al.*, 2009a).

Upon comparison of root proteomes, it is striking that in long term Cd exposure the carbohydrate metabolism was affected by 45.9% of the differentially abundant proteins whereas it was only affected by 19% after short term exposure. Interestingly, after short term exposure the carbohydrate metabolism was exclusively up-regulated, whereas it was mainly down-regulated after long term exposure. The effect on carbohydrate metabolism after short term Cd exposure can almost exclusively be ascribed to an up-regulation of catabolism. Here, glyceraldehyde-3-phosphate dehydrogenase was up-regulated, producing NADH, which could be necessary in the detoxification of oxidative stress (see section 4.2.6.1.1.1). In contrast, changes in carbohydrate metabolism after long term exposure were mainly due to down-regulation of proteins involved in catabolism. Looking more into detail reveals that these proteins were mainly involved in the TCA cycle and glycolysis. This down-regulation is also in contrast to the response in leaves where an up-regulation of catabolism was seen after long term exposure (see section 4.2.6.1.1.1). All described up-regulated catabolic proteins in long term Cd-exposed roots were reported by Rodríguez-Celma *et al.* (2010) and therefore this up-regulation might be a specific response to low Cd concentration, as used by these authors. Data presented in

this meta-analysis confirmed the down-regulation of the carbohydrate metabolism described in tomato roots after long term Cd exposure (Rodríguez-Celma *et al.*, 2010).

Stress metabolism on the other hand, was affected by 42.9% of all differentially abundant proteins after short term exposure and only for 21.8% after long term exposure. Thus indicating a lower stress response after long term exposure, contrasting with the effects found on leaf proteomes. The same pattern was described by Kieffer *et al.* (2009b). Similar to what is observed in leaves, the effect on stress metabolism after short term exposure was mainly due to up-regulation of proteins involved in detoxification. Long term stress response also mainly affected detoxification proteins. However here a more or less equal distribution between up-/down-regulation was present. Upon long term exposure, proteins involved in GSH metabolism were up-regulated whereas proteins related to ascorbate metabolism were down-regulated, which suggests that GSH is involved in multiple detoxification strategies.

In order to maintain cellular function, to respond to a specific stress and to recycle damaged macromolecules, nucleotide and protein metabolism must be addressed (Semane *et al.*, 2010). After short term Cd exposure, up-regulation of some proteins involved in nucleotide metabolism was present. In contrast, proteins involved in nucleotide metabolism were exclusively down-regulated after long term Cd exposure. Focusing on protein metabolism revealed an up-regulation of protein synthesis and degradation after short term exposure. After long term exposure, protein synthesis was clearly down-regulated whereas protein (re)folding was up-regulated. Since glutamine and cysteine are important precursors in GSH biosynthesis, it is somewhat surprising that proteins involved glutamine and cysteine synthesis were down-regulated upon long term Cd exposure. Up-regulation of proteins involved in protein folding was probably due to the strong affinity of Cd for thiol groups, resulting in the destabilization of proteins (e.g. by cleaving disulfide bridges; Ashan *et al.*, 2009). The combined data in this meta-analysis confirmed the increase in protein (re)folding reported by both studies on long term Cd-exposed roots (Kieffer *et al.*, 2009b; Rodríguez-Celma *et al.*, 2010).

4.2.5.1.2. Soil cultivation system – Short term versus long term cadmium exposure

No manuscript was found studying the proteome of the leaves of soil grown plants during short term Cd exposure. This indicates an overall agreement on the presence of a delayed Cd effect in leaves for plants grown in soil cultivation systems. Comparing the total number of differentially abundant proteins (DAPs) in hydroponically grown roots after short term exposure (21 DAPs) to those of soil grown roots after short term exposure (162 DAPs) reveals major proteomic changes in soil grown plants upon short term exposure and therefore it might be worthwhile to explore the short term cadmium exposure on leaves in soil grown plants as well! Since no studies have focused on the leaf proteome of soil grown plants after short term Cd exposure, no comparison can be made for leaves.

4.2.5.1.2.1. Root proteome

Up to date only 1 study focused on the effect of Cd on root proteins in a short term experiment (Alvarez *et al.*, 2009; Table 4.2.3). In this study, 162 proteins were reported to differ in abundance, 76 were up-regulated and 86 were down-regulated. Two articles focused on root proteins of plants grown in soil cultivation systems upon long term Cd exposure (Repetto *et al.*, 2003; Aloui *et al.*, 2009; Table 4.2.3). Focusing on long term exposure, only 21 proteins differed in abundance. Of them, 17 were up-regulated and 4 were down-regulated (Table 4.2.4). Considering total DAPs, one could conclude that more proteins in roots are affected upon short term exposure compared to long term exposure. This idea is in line with the fact that the root is the first organ to come into contact with Cd. Subsequently, Cd is frequently translocated in the aerial parts of the plant (Ashan *et al.*, 2009; Kieffer *et al.*, 2009a).

Upon short term exposure, the protein metabolism was primarily affected (28.4%) in root proteome of soil grown plants, followed by the stress metabolism (23.5%). After long term exposure, the stress metabolism was mainly affected, followed by the carbohydrate metabolism, together covering more than 60% of all DAPs.

The high effect on protein metabolism after short term exposure, was due to a high down-regulation in protein metabolism described using the iTRAQ technology (Alvarez *et al.*, 2009). Here, 75% of all down-regulated proteins

were involved in protein metabolism. As reported by the authors, huge discrepancies were present comparing iTRAQ analysis to 2D-DIGE analysis. Focusing only on 2D-DIGE, Alvarez *et al.* (2009) would underestimate the importance of the protein metabolism upon short term Cd exposure. This suggests that, in order to get a full understanding of the effect of Cd on the plants proteome, different complementary techniques should be used in proteomic studies unravelling the Cd effect on plants (as discussed in 4.2.2.2.1). After short term exposure, three proteins involved in cysteine synthesis were up-regulated suggesting their involvement in Cd stress response via GSH synthesis. The down-regulation of ribosomal protein synthesis may indicate a redeployment of resources to meet the greater demand for amino acids in the non-ribosomal peptide synthesis of GSH and phytochelatins, needed for Cd complexation (Alvarez *et al.*, 2009; Jozefczak *et al.*, 2012; Seth *et al.* 2012). Upon long term exposure, only two proteins involved in protein metabolism were reported to be differentially abundant and both are down-regulated. Cyclophilin is classified under 'protein folding' since this is reported as its basic function, however it might be involved in mRNA processing, protein degradation and signal transduction (Romano *et al.*, 2004). The second protein is involved in regulation of 26S proteasome and is therefore classified under protein degradation.

Compared to long term exposure, stress metabolism was only moderately affected upon short term exposure (23.5%). Considering up-regulated stress proteins after short term exposure, defence proteins were higher addressed than detoxification proteins. An overall increase in both GSH and ascorbate-related detoxification proteins was present. In addition, some ROS-related stress proteins were down-regulated as well as some defence related proteins such as an endochitinase and a thioglucoside glucohydrolase (Kumar *et al.*, 2009; Husebye *et al.*, 2002). After long term exposure stress metabolism was exclusively up-regulated, accounting for 42.9% of all differentially abundant proteins in this conditional subgroup. As after short term exposure, defence proteins were higher addressed than detoxification proteins.

Focusing on energy metabolism, a mitochondrial ATP synthase and an ubiquinol cytochrome C reductase-like protein were up-regulated upon short term exposure while no effect on energy metabolism is reported after long term

exposure. The up-regulation of ATP synthase might indicate a stimulation of energy production via mitochondrial respiration. Upon short term exposure, catabolism and more specifically glycolysis was down-regulated while an up-regulation of proteins involved in the metabolism of C-based molecules was present.

To conclude this comparison, it should be mentioned that some proteins were reported to be down- as well as up-regulated (see Table S1; supplementary data), which can possibly be explained by post-translational modifications.

4.2.5.2. Culture comparison

As stated above, hydroponic cultivation systems are often used in proteomic studies. Since they offer poor comparability to field conditions, it is interesting to know whether proteomic data obtained in hydroponic cultivation systems is comparable to those obtained in soil cultivation systems, which give a more realistic representation of the field. Therefore, the effect of Cd upon leaf and root proteomes will be compared between hydroponic and soil cultivation systems in this section.

In hydroponically grown leaves, CO_2-fixation was down-regulated upon short and long term Cd exposure. In soil grown leaves, the down-regulation of CO_2-fixation was confirmed upon long term Cd exposure, however it could not be confirmed after short term Cd exposure since no study was conducted on soil grown leaves after short term exposure. In roots, the energy metabolism appeared to be almost unaffected, while energy production was clearly down-regulated in both hydroponically and soil grown leaves after long term Cd exposure. Consequently, a metabolic shift to a lower carbon fixation and a higher respiration was present in leaves after long term Cd exposure, independent of the cultivation system. Glycolysis was up-regulated in leaves and down-regulated in roots upon long term Cd exposure, independent of the cultivation method. However, glycolysis was highly affected in hydroponically grown leaves and roots while only mild responses were present in soil grown leaves and roots. Next to the observed similarities in differentially abundant proteins between hydroponic and soil cultivation systems, some contradictions were present as well. Focusing on stress metabolism, proteins involved in detoxification were mainly addressed in hydroponic cultivation systems whereas

proteins involved in defence were mainly addressed in soil cultivation systems. In hydroponic cultivation systems, several NADH-producing proteins were up-regulated in roots after short term exposure. Upon long term exposure, the up-regulation of NADH-producing proteins shifted from the roots, where now a high down-regulation of NADH-producing proteins was present, towards the leaves. As stated above, the production of NADH is important for the detoxification of Cd-induced ROS (Kieffer *et al.*, 2009a). Although no NADH-producing proteins were present in soil grown roots upon long term exposure, the shift towards the leaves could not be confirmed since only three NADH-producing proteins differed in abundance, of which two were up-regulated and one was down-regulated. On top, only one NADH-producing protein was up-regulated in roots after short term Cd exposure while two were down-regulated under the same conditions.
However, based on the data represented by this meta-analysis, it is hard to postulate whether or not extrapolations from hydroponic cultivation systems towards field experiment is possible since a huge variety in experimental setup among the included studies is present. Ideally, to test the influence of the cultivation system on the plants' proteome upon stressed conditions, a large scale experiment including hydroponic and soil cultivation systems should be conducted keeping all remaining parameters identical.

4.2.6. General conclusion

With the prospect on boosting phytoextraction efficiency of Cd-contaminated soils, we wanted to perform a meta-analysis of plant proteome studies focussing on Cd stress. Having insights in molecular mechanisms that are addressed upon Cd stress, might provide researchers with tools to boost phytoextraction efficiency. Exploring the literature, we quickly stumbled on several obstacles making a meta-analysis quite challenging. To start, a huge diversity on material and methods is present in the 33 articles focussing on plants' responses to Cd stress. Differences in cultivation methods and exposure make comparison very hard. On the other hand, differences in the proteomic workflow are believed to be complementary to each other. For that reason, no distinction was made between proteomic techniques. However, we want to alert proteomic scientists that there is a need for more detailed information on proteomic studies and that general guidelines and a public repository of raw data are indispensable. Since

its beginning in the 70s by the development of 2D-PAGE (Kenrick and Margolis, 1970), proteomics has been used in various studies and has undergone several changes and adaptations in order to improve the techniques and the general workflow. The original goal of proteomics was to characterize and evaluate the whole proteome of a cell, tissue or organism (Lemos *et al.*, 2010). However, it is generally accepted that, with the techniques available to date, we are only able to study sub-proteomes and therefore, up till now the main goal of proteomics remains an illusion. As stated by Lemos *et al.* (2010), the main question is 'how can one determine the full proteome of an organism' and the answer is simple, although shocking for the least aware: 'you cannot'. Proteomic researchers need to be aware of the fact that the generated proteome is highly dependent on the chosen proteomic workflow and should keep this in mind when setting up their experimental goals. Another important note is that there is no cross laboratory standardization making comparisons of similar studies in different labs difficult. Being a quite complex technology, proteomics is typified by a specialist in a specific domain. These specialists often conduct studies to further optimize the proteomic workflow. Being a non-specialist, it becomes more and more difficult to get the whole picture. As a result, proteomic data will be poorly reported and/or misinterpreted. On top, we'd like to point out that, although high throughput proteomics is necessary to obtain a total proteome response, many researchers find it difficult to extract biologically relevant information from large datasets. Therefore we believe general guidelines are necessary in the proteomic field to render good validate proteomic results and to avoid "black box" approaches, since it is impossible for a researcher to be specialised at each level of the proteomic workflow. Despite the encountered obstacles, the meta-analysis on the plants' response to Cd revealed some interesting ideas which will be discussed hereafter.

To start, based on the total DAPs (Table 4.2.6), leaf proteome changed more drastic upon short term exposure in hydroponic cultivation systems compared to root proteome under the same conditions. This might seem contradictory to the general accepted 'delay effect' which postulates that leaves are later affected by Cd exposure since Cd enters at root level and needs some time to reach the leaves and accumulate herein (Kieffer *et al.*, 2009a). However, it should be kept in mind that it is difficult to preconceive a threshold to classify 'short' and 'long'

term exposure since this highly depends on the plants' metabolic speed. Only one study compared leaf and root proteomes upon short term exposure (Kieffer *et al.*, 2009b) and observed that after 3 days of exposure, control and Cd gels of leaf proteomes could not be separated, while root extracts showed a clear separation after 3 days of Cd exposure. In order to get a better idea of the existence of a 'delay effect' on plants' proteome after Cd exposure, more large scale studies should be conducted comparing leaf and root proteomes upon short term Cd exposure.

A new equilibrium seems to be established in soil grown roots after long term exposure since substantial less proteins were differentially abundant after long term exposure; 21 DAPs versus 162 DAPs after short term exposure. This confirms the idea that Cd is considered to induce an initial 'alarm phase' where after a stabilization of the stress response is described (Lichtenthaler, 1996; Kieffer *et al.*, 2009). However, the effects on stress metabolism contradict this since it was highly up-regulated after long term exposure, accounting 40.9% of all DAPs. This indicates that stabilization might occur at metabolisms other than the stress metabolism. Hydroponically grown plants appeared to be unable to establish an equilibrium after long term exposure since more DAPs were reported upon long term exposure in both leaves and roots compared to short term exposure. This might be explained by the fact that in hydroponic cultivation systems a homogeneous distribution of the contaminant is present and roots did not have the chance to grow towards less contaminated areas as they do in soils (Remans *et al.*, 2012). Furthermore, a closer look into two studies that focused simultaneously on low and high Cd exposure (Rodríguez-Celma *et al.*, 2010; Semane *et al.*, 2010) pointed towards a moderate Cd effect on the proteome when low concentrations were applied. This moderate effect is thought to be due to Cd-induced Fe deficiency (Rodríguez-Celma *et al.*, 2010) and is seen as an attempt to maintain cellular function (Semane *et al.*, 2010).

Finally, the carbohydrate metabolism is primary affected in leaves, independent of the exposure time and the cultivation system, confirming the findings of Kieffer *et al.* (2009b). On top, the metabolic shift from CO_2-fixation towards respiration is manifested predominantly in long term exposed leaves, independent of the used cultivation system.

Comparing the data presented by this meta-analysis to the general Cd effects in plants proposed by Villiers *et al.* (2011) confirmed (1) drastic changes at the RuBisCO level, (2) the need to remobilize energy and reductive power from other metabolic pathways and (3) the expression of several enzymes involved in oxidative stress defence and detoxification. Presenting a summary of proteomics and metabolomics, Villiers *et al.* (2011) proposed a working model on plants' response to Cd. However, data presented in this meta-analysis however could not confirm the presented working model when looking at the different conditional groups (Table 4.2.7). This is probably due to the fact that cell suspension cultures, leaf segments floating in Cd solution and seeds were included in the review of Villiers *et al.* (2011). Additionally, based on the proteins involved in the model, a difference between hydroponic and soil cultivation systems was present (Table 4.2.7). Furthermore, it should be mentioned that some proteins involved in the calvin cycle are reported to be both up- and down-regulated by Durand *et al.* (2010). Although this might be explained by post-translational modifications, it prevents a clear conclusion on the up- or down-regulation of the calvin cycle in soil grown leaves upon long term exposure.

Focusing on Cd exposure, *in situ* phytoextraction is often a future prospect. Although promising, phytoextraction still suffers from several disadvantages. One major disadvantage is the large time scale necessary to clean up contaminated soils. In order to overcome this disadvantage, several researchers focus on the underlying mechanisms induced by Cd stress. Unravelling these underlying mechanisms and understanding the effects Cd induces on the plants' metabolism, scientists hope to boost phytoextraction efficiency. As described by Rossignol *et al.* (2006), a better knowledge of the plant proteome, as a way of understanding phenotypic plasticity and adaptability in plants, is required to effectively exploit plant biological resources. However, while field application is clearly a future prospect, most studies unravelling the Cd response use hydroponic cultivation systems to cultivate their plants, even though suggestions about the differences induced by cultivation systems are present (Durand *et al.*, 2010). Although Lombi *et al.* (2000; hyperaccumulators) and Watson *et al.* (2003) stated that the results obtained from hydroponically and soil grown

Table 4.2.7: Comparison to the working model proposed by Villiers *et al.* (2011). For each conditional subgroup, proteins involved in the working model of Villier *et al.* (2011) are shown to be increased (green) or decreased (red).

	Abbrevia-tions	VILLIERS *et al.* 2011	Hydroponic cultivation system				Soil cultivation system			
			SHORT		LONG		SHORT		LONG	
		Response	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
Sulphur assimilation, amino acid synthesis										
ATP sulphurylase	APS						No reported proteins			
APS reductase	APR									
Serine acetyl transferase	SAT									
O-acetylserine(thiol)lyase	OAS									
Serine hydroxymethyltransferase (cytosolic and mitochondrial)	SHMT									
Glutamine synthetase	GLN1									
Glutamate synthase	GLT									
Photosynthesis, primary target of Cd										
Ribulose-1,5-bisphosphate carboxylase/oxygenase	RuBisCO						No reported proteins			
Calvin cycle										
Photosystem II	PSI									
Cytochrome b6f	cyt b6f									
Photosystem I	PSII									
Ferredoxin-NADP reductase	FNR									
Detoxification and compartmentalization										
Gamma-glutamylcysteine synthetase	GSH1						No reported proteins			
Gamma-glutamylcysteine										
Glycine										
Glutathione synthetase	GSH2									
Phytochelatin synthase1	PCS1									
Phytochelatin synthase	PCS2									
Glutathione S transferases	GSTs									
Iso-phytochelatins	isoPC									
Phytochelatins	PC									
Selenium binding protein 1	SBP1									
Cation eXchanger	CAX1a									
Multidrug Resistance Protein Like	MRP LIKE									
Natural resistance-associated macrophage protein 3	NRamp 3									
Natural resistance-associated macrophage protein 4	NRaMP 4									
Carbohydrate metabolism										
Alpha amylase							No reported proteins			
Hexose kinase	HK									
Phosphoglucose isomerase	PGI									
Fructokinase	FK									
Aldolase	ALD									
Triosephosphate isomerase	TPI									
Cytosolic glyceraldehyde 3 phosphate dehydrogenase	GAPC									
Phosphoglycerate kinase	PGK									
Phosphoglycerate mutase	PGM									

Table 4.2.7: Comparison to the working model proposed by Villiers *et al.* (2011). For each conditional subgroup, proteins involved in the working model of Villier *et al.* (2011) are show to be increased (green) or decreased (red) (Continued).

	Abbrevia-tions	VILLIERS *et al.* 2011	Hydroponic cultivation system				Soil cultivation system			
			SHORT		LONG		SHORT		LONG	
		Response	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
Carbohydrate metabolism										
Enolase / 2phosphoglycerate hydrolase	ENO						No reported proteins			
Pyruvate kinase	PK									
Glucose6phosphate dehydrogenase	G6PDH									
6-phosphogluconolactonase	6PGL									
Phosphogluconate dehydrogenase	PGDH									
Ribulose phosphate 3 epimerase	RP3E									
Phosphate isomerase	R5PI									
Transketolase	TK									
Pyruvate dehydrogenase	PDH									
Malate dehydrogenase	MDH									
Succinate dehydrogenase	SUC									
NADP-specific isocitrate dehydrogenase	ISO									
Aconitase	ACO									
Citrate synthase	CS									
Proteolysis										
Ubiquitine E1	UbE1						No reported proteins			
Ubiquitine E2	UbE2									
Ubiquitine E3	UbE3									
Proteasomes										
Tripeptidyl peptidase II	TPPII									
Thimet oligopeptidase	TOP									
Aminopeptidases										
Oxidoreduction control										
Glutathione reductase							No reported proteins			
Catalases										
NADPH oxidoreductase										
L-ascorbate peroxidase										
Monodehydroascorbate reductase										

plants were similar, data presented by this meta-analysis confirm that differences between hydroponic and soil cultivation systems might exist, suggesting that extrapolation from hydroponic cultivation systems to field conditions might be not that straightforward. However, since the meta-analysis is based on studies with a high diversity in the experimental setup, this hypothesis cannot be confirmed or rejected yet. Therefore, there is a need for large-scale studies addressing the influence of the cultivation systems on the Cd proteome response.

4.2.7. Perspectives

Most studies on the effect of Cd on the plants' proteome focus on (1) leaves or roots, (2) only one Cd concentration and (3) only one exposure time. Of the included studies, only two research groups focused on a time scale effect of Cd (Kieffer *et al.*, 2008, 2009a, 2009b; Fagioni and Zolla, 2009a), only two on two different Cd concentrations (Semane *et al.*, 2010; Rodriguez-Celma *et al.*, 2010) and only one focused simultaneously on roots and leaves (Kieffer *et al.*, 2009b). To get a better idea of the overall plants' response to Cd, there is an urgent need for large-scale experiments. Since Cd toxicity depends on the applied concentration (Kacperska *et al.*, 2004; Smeets *et al.*, 2005; Rodríguez-Celma *et al.*, 2010), the exposure time (Smeets *et al.*, 2005) and the plant species (Rodríguez-Celma *et al.*, 2010), the large-scale studies should be conducted focussing on these aspects. Additionally, since most Cd studies on plants are conducted with a future prospect on phytoextraction, we question if future research should focus on plant species relevant for phytoextraction e.g. poplar, willow, maize. On the other hand, researchers should be aware that species which are interesting for us as scientists because of fast growth, easy cultivation, known genome, ... are often unusable for farmers.

Although proteomics is believed to be a promising technique since proteins are the functional units in a cell that are responsible for most biological functions, it should be integrated in the total 'omics' approach. Not only is there no correlation between the amount of mRNA and the protein concentration, protein concentration and activity can be altered by modifications (pre-modification, co-modifications, post-translational modifications), by the concentration of regulators in the cell and by the rate of protein degradation (Lemos *et al.*, 2010). Since there are over 300 different types of protein modification described in the literature (Garavelli, 2004) and since on average every eukaryote protein might have eight to ten post-translational variants (Barret *et al.*, 2005), it is easy to understand that only the insights in differential protein expression are too little to understand the response of the plant. Consequently, a full systems biological approach is desirable for a complete understanding. Furthermore, experiments investigating the effect of Cd on the plants' proteome usually use classical gel-based proteome techniques. The use of new generation proteomic techniques is still in preliminary stage in this research field. On top, up to date

very few research groups worked with fractionated samples, although this is preferred to get a better understanding of the plants' total response to Cd, since a larger part of the total proteome can be separated, analyzed and finally, identified.

Chapter 4.3

Effects of short and long term Cd exposure on the proteome of roots and leaves of hydroponically grown poplar (P. deltoides x (trichocarpa x deltoides))

4.3.1 Introduction

To unravel physiological mechanisms involved in stress responses, monitoring molecular dynamics of an organism upon stress has been proposed to be one of the best approaches (Villiers *et al.*, 2011). Quantitative analysis of proteins and metabolites can provide reliable information about molecular changes, allowing to indicate a range of more or less specific pathways involved. In addition, proteomic analysis could lead to the identification of major players in the considered pathways (Villiers *et al.*, 2011). Over the last ten years, an increase in plant proteomic studies comparing control versus Cd-exposed conditions was present (see Table 4.2.1). Despite this increased availability of proteomic data, it yet remains hard to establish a clear overview of the cellular responses induced by Cd. As demonstrated in the meta-analysis (See section 4.2.6), it is hard to draw conclusions between reported data with different setups. Moreover, it has been questioned whether or not heavy metal detoxification mechanisms accord with the 'General Adaptation Syndrome (GAS)' hypothesis, characterized by the fact that different types of stresses evoke similar or even identical stress coping mechanisms (Leshem and Kuiper, 1996; di Toppi and Gabrielli, 1999). As stated above, large scale studies need to be conducted to get better insights in the plants' response to Cd.

In order to focus on Cd-specific effects on the plants' proteome, variations of other environmental factors need to be minimized as much as possible. A hydroponic cultivation system was chosen for this experiment since it provides highly controllable conditions and reduces the variability due to environmental factors (Zacchini *et al.*, 2009). To identify early and long term changes due to Cd exposure, sampling was done at respectively 3 and 21 days after start of the exposure. Although a comparable study was already done by Kieffer *et al.* (2009b) on *Populus tremula* L., this experiment will provide additional data since

the relative importance of each response may differ for plant species (di Toppi and Gabrielli, 1999). Being part of a large scale research study on phytoextraction, molecular insights revealed by this study will contribute to a better understanding of the effects of Cd on *Populus deltoides x (trichocarpa x deltoides)*, which might tackle the 'black box' approach of using this species in phytoextraction trials.

4.3.2 Materials and methods

4.3.2.1 Plant cultivation: Hydroponic cultivation system

Poplar cuttings (*Populus deltoides x* (*trichocarpa x deltoides*)) of 30 cm were grown in hydroponic systems (2 liter, aerated solution; Figure 4.3.1). Plants were incubated in a growth chamber at day/night temperature of 22/18°C with a 12 h light period, relative humidity of 65% and photosynthetic active radiation of 170 µmol m^{-2} s^{-1}. For the next weeks, hydroponic solutions were refreshed with ½ strength Hoagland's solution (Hoagland and Arnon, 1938) once every weeks and the water level was replenished to the maximum level by adding distilled water three times a week. Once sufficient fully expanded leaves were present on all cuttings, cuttings were divided into four batches:

- Batch 1: control group, harvested after 3 days of Cd exposure (control group of batch 2)
- Batch 2: Cd group, harvested after 3 days of Cd exposure
- Batch 3: control group, harvested after 21 days of Cd exposure (control group of batch 4)
- Batch 4: Cd group, harvested after 21 days of Cd exposure

Cd groups were exposed to 20 µM $CdSO_4$. After start of the exposure, the same culture schedule was applied but Cd groups were refreshed with ½ strength Hoagland's solution supplemented with 20 µM $CdSO_4$. At day 3 and day 21, the according batches were sampled. Of each batch, growth parameters were measured, 5 leaf and root samples were taken for protein analysis and 5 leaf and root samples were taken to determine tissue Cd content. Five samples of the hydroponic solution of each batch were taken to determine total Cd content.

Using hydroponic cultivation systems, Smeets *et al.* (2009) mentioned some critical points that should be considered: (1) variations induced by the hydroponic cultivation systems should be taken into account, (2) sampling

should follow an appropriate protocol to prevent time-born effects or within-pot correlations, (3) the nutrient solution should be refreshed daily and external fluctuations should be avoided and finally, (4) the nutrient solution should be continuously aerated. In preliminary experiments, hydroponic cultivation systems using poplar cutting were tested, revealing that poplar roots are very sensitive and difficult to handle. Therefore it was required to develop a hydroponic cultivation system that allows nutrient refreshing without disturbing the poplar roots (Figure 4.3.1).

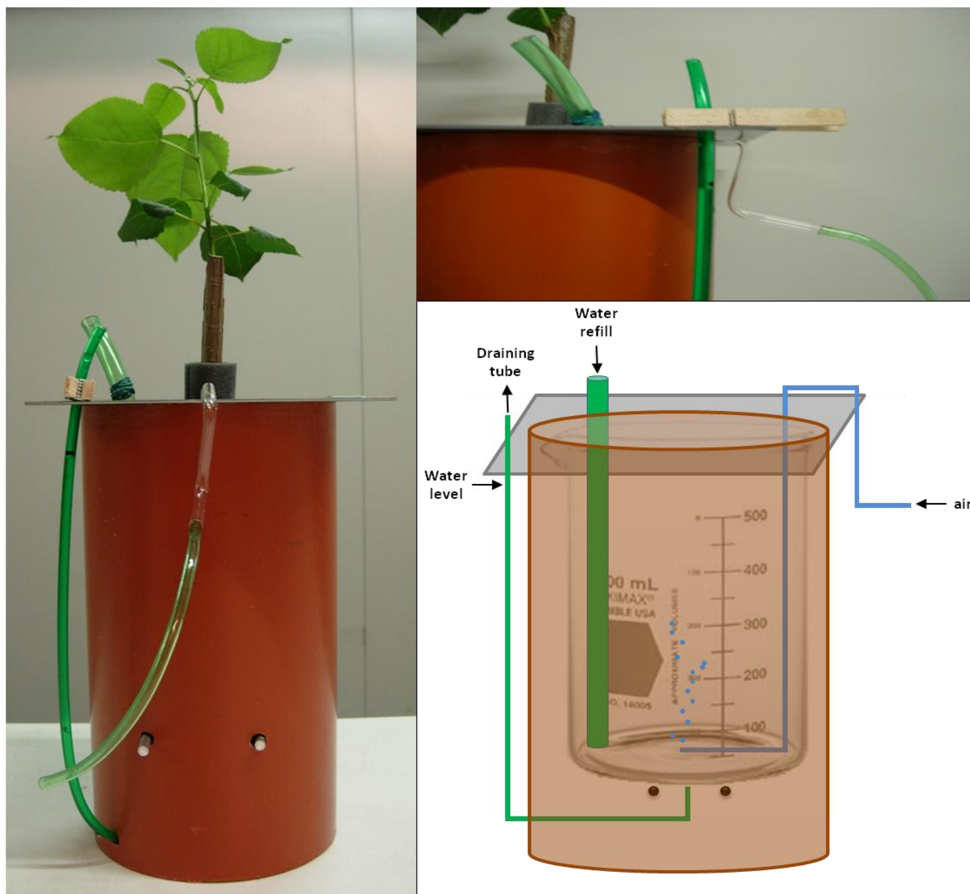


Figure 4.3.1: Hydroponic cultivation system. Water level was maintained by using the draining tube as a communicating vessel system; water was refilled bottom-up using a tube; air was applied using a glass aerating tube at the middle of the soil. Roots were kept in the dark at all times and were not manipulated during the experiment.

Test running this novel system revealed that a daily refreshing of the nutrient solution is not ideal for poplars. Since leaf chlorosis was observed in case the nutrient solution was refreshed daily, a weekly refreshment was tested. The latter allowed poplar cuttings to grow homogeneously and moreover, no visible stress symptoms were present. Further, this novel cultivation system also allows continuous aeration of the nutrient solution. In respect with the critical points mentioned by Smeets *et al.* (2009), all samplings were performed within the hour to limit temporal changes. Furthermore, leaves of the same age were sampled and root samples consisted of several entire adventitious roots.

Growth parameters were statistically analyzed using a two-way ANOVA (condition 1: time, condition 2: exposure; $p<0.05$) and *post hoc* multiple comparison testing (Tukey Kramer). When necessary, log-transformations were applied to approximate normality and/or homoscedasticity. Statistical analyses were performed using SAS 9.2.

4.3.2.2 Analysis of metal content in plant tissue and hydroponic solution

4.3.2.2.1 Metal content in plant tissue

Each fresh sample (5 leaf and root samples per batch; see section 4.3.2.1) was dried at 60°C. Samples were crushed and 150 mg DW was taken for analysis. To each sample, 2 ml HNO_3 suprapur was added and samples were stored overnight at room temperature. Subsequently, samples were heated to 110°C in order to evaporate the remaining HNO_3 suprapur. 1 ml HNO_3 suprapur was added and evaporated again. Thereafter, samples were dissolved in 1 ml HCl suprapur and heated in a final step to evaporate the HCl suprapur. Finally, samples were dissolved in 20% HCl and measured using flame atomic absorption spectrometry (AAS) to determine Cd, Zn and Fe content.

4.3.2.2.2 Metal content in hydroponic solution

Of each batch, 5 samples of 50 ml hydroponic solution was taken and analysed by AAS to determine Cd, Zn and Fe content.

4.3.2.2.3 Statistical analysis

Metal contents were statistically analyzed using a two-way ANOVA (condition 1: time, condition 2: exposure; $p<0.05$) and *post hoc* multiple comparison testing (Tukey Kramer). When necessary, log-transformations were applied to

approximate normality and/or homoscedasticity. Statistical analyses were performed using SAS 9.2.

4.3.2.3 Protein extraction

Plant tissue was grinded in liquid nitrogen. Per gram fresh weight (FW) 10 volumes of ice cold acetone were added. After 1 h precipitation at -20°C, samples were centrifuged (10 000 g, 10 min, 4°C), the pellet was freeze-dried, and 10 volumes of extraction buffer (50mM Tris pH 8.5, 25 mM Na_2EDTA, 100 mM KCl, 30% w/v sucrose, 2% β-mercapto-ethanol and 0.4 mM PMSF) were added per gram FW. Ten minutes later, the same volume of phenol was added and the solution was mixed thoroughly. After centrifugation (8000 rpm, 5 min, 4°C) the phenolic phase was collected, 5 volumes of ammonium acetate in proportion to the volume of collected phenol, were added and the sample was allowed to precipitate overnight at -20°C. After centrifugation (10 000 g, 10 min, 4°C) pellets were washed three times with ammonium acetate and once with DTT/acetone (0.2% w/v). Finally, pellets were resuspended in resuspension buffer (7 M urea, 2 M thio-urea, 4% CHAPS and 30 mM TRIS), incubated at 18°C (1200 rpm, 2 h) and centrifuged (70 000 g, 90 min, 18°C) to remove final interfering substances. Prior to storage at -80°C, samples were brought to pH 8.5 using 0.5 M NaOH.

4.3.2.4 Protein quantification

Protein samples were quantified using the EZQ protein quantitation kit (Molecular Probes). Standard protocol was used with some minor modifications. A serial dilution ranging from 0 – 1 mg ml^{-1} was prepared to make a standard curve. Samples were 1/20 diluted in resuspension buffer and analysed in triplet. Two microliter of each sample and dilution series was loaded to minimize pipetting errors. Ovalbumine was used as a reference.

4.3.2.5 DIGE labelling

Protein samples were analysed by fluorescence difference in gel electrophoresis (DIGE). Prior to electrophoresis, protein extracts and a pooled standard were labelled with CyDyes™ (GE healthcare). The pooled internal standards, used to normalize gels, were prepared separately for leaf and root samples. Therefore,

an equal protein quantity of each leaf (or root) sample was pooled. All leaf (or root) samples were randomized for labelling with Cy3 and Cy5 (Table 4.3.1), whereas pooled standards were labelled with Cy2. All samples were labelled at a ratio of 240 pmol protein minimal labelling dye for 30 µg of protein. Labelled samples were vortexed and incubated on ice for 30 min in the dark. To quench reactions, 1 µl of 10 mM lysine was added, samples were vortexed and incubated on ice in the dark for 10 min. Next, 90 µg of proteins was loaded onto each gel and separated by 2-DE: 30 µg Cy2-labelled internal standard, 30 µg Cy3-labelled sample and 30 µg Cy5-labelled sample. Samples were labelled in a randomized setting.

Table 4.3.1: Schematic overview of randomization of protein samples. In total, 10 leaf and root samples were labelled by CY3 and 10 leaf and root samples were labelled by CY5.

Condition	**Leaf samples**		**Root samples**	
	Sample ID	Labelling CyDye	Sample ID	Labelling CyDye
BATCH 1	1	Cy3	1	Cy3
	2	Cy3	2	Cy3
	3	Cy3	3	Cy3
	4	Cy5	4	Cy5
	5	Cy5	5	Cy5
BATCH 2	6	Cy3	6	Cy3
	7	Cy3	7	Cy3
	8	Cy5	8	Cy5
	9	Cy5	9	Cy5
	10	Cy5	10	Cy5
BATCH 3	11	Cy3	11	Cy3
	12	Cy3	12	Cy3
	13	Cy5	13	Cy5
	14	Cy5	14	Cy5
	15	Cy5	15	Cy5
BATCH 4	16	Cy3	16	Cy3
	17	Cy3	17	Cy3
	18	Cy3	18	Cy3
	19	Cy5	19	Cy5
	20	Cy5	20	Cy5

4.3.2.6 2-DE

For the IEF, 24 cm strips with a non-linear pH gradient ranging from 4 to 7 (GE Healthcare) were used in the IPGphor system (Amersham Biosciences, Uppsala, Sweden). The strips were rehydrated overnight in rehydration buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 65 mM DTT, 0.8% v/v IPG buffers, 0.002% v/v bromophenol blue) containing proteins. IEF was carried out on an Ettan IPGphor Manifold (GE Healthcare) with the following settings: constant step of 50 V during 100 Vhrs, constant step of 150 V during 300 Vhrs, constant step of 300 V during 600 Vhrs, gradient step of 1000 V during 3900 Vhrs, constant step of 1000 V during 1000 Vhrs, gradient step of 8000 V during 18000 Vhrs and a final constant step of 8000 V during 32100 Vhrs, for a total of 56000 Vhrs. IEF was performed at 20°C with a maximum current setting of 50 µA/strip. On the paper wicks at the negative electrode, 150 µl DeStreak rehydration solution (GE Healthcare) was added to reduce streaking. Paper wicks were replaced after the first focusing step. After the IEF, IPG strips were equilibrated at 18°C for 15 min in equilibration buffer (75 mM Tris pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS and a trace of bromophenol blue) supplemented with 1% w/v DTT. A second equilibration step of 15 min with the same equilibration buffer, now containing 2.5% w/v iodoacetamide, was carried out afterwards. Next, IPG strips were sealed on top of 200mm x 260mm x 1mm, 12.5% polyacrylamide gels with 0.5% agarose in SDS running buffer. Gels were poured between low fluorescent glass plates, of which one plate was bind-silane treated. The SDS-PAGE step was performed at 15°C in Ettan Dalt II tanks (GE Healthcare) at 1.4 W per gel for 17 h. To produce Cy2-, Cy3- and Cy5-labelled protein images, excitation of gels at 488, 532 and 633 nm respectively was performed on an Ettan DIGE Imager (GE Healthcare).

4.3.2.7 Analysis

Images were analyzed using the Decyder v7.0.8.35 software (GE Healthcare). Spots of interest were selected based on an absolute abundance variation of at least 1.5-fold (ANOVA; $p < 0.05$). Picking gels were generated using a mixture containing 5 µg of all leaf (or root) samples. After loading this mixture onto the gel, gels were subjected to the same workflow as the analytic gels (section 4.3.2.6). Subsequently, gels were stained using LavaPurple™ according to the

manufactures' guidelines and picking lists were generated. Proteins of interest were excised using the ProPic II spotpicker (DIGILAB).

4.3.2.8 Digestion and identification

Excised spots were immediately submerged into 100% acetonitrile. When dry, they were stored at -20°C prior to digestion. To digest, 30 µl 10 mM DTT in 100 mM NH_4HCO_3 was added to the samples. After an incubation of 1 h at 56°C, samples were cooled to room temperature, supernatant was removed and 30 µl of 55 mM IAA in 100 mM NH_4HCO_3 was added. After an incubation of 45 min in the dark, supernatant was removed and samples were washed with 100 µl of 100 mM NH_4HCO_3. Thereafter samples were dehydrated using 100 µl 100% acetonitrile. When washing and dehydration were repeated, samples were dried in a vacuum centrifuge (Concentrator 5301, Eppendorf). Next, samples were incubated on ice and 10 µl trypsin (12.5 ng μl^{-1}) digestion buffer was added. After an incubation period of at least 3 hours, supernatant was removed. After an overnight incubation at 37°C, the supernatant was collected. To extract remaining peptides, 20 µl 20 mM NH_4HCO_3 was added, samples were sonicated (Branson 5210, Gemini) for 20 min and supernatant was collected. Next, 50 µl of 5% formic acid in 50% acetonitrile were added to the samples. After a sonication of 20 minutes, the supernatant was collected and this step was repeated twice. Finally samples were dried in a vacuum centrifuge and stored at -20°C prior to identification.

Tryptic digests were analysed by LC-ESI-MS/MS on a LCQ Classic (Thermo Electron, San Jose, CA, USA) ion trap mass spectrometer equipped with a nano-LC column switching system as described by Dumont *et al.* (2004). MS/MS data were searched against the *Populus trichocarpa* v1.1 protein database (87596 entries) using Mascot (version 2.04.0; Matrix Sciences, London, UK) and Sequest (version 1.2.0.208 within Proteome Discoverer version 1.2; Thermo Fisher Scientific). Sequest and Mascot parent ion mass tolerance was set to 3 Da; fragment ion tolerance was 1 Da. Carbamidomethylation of cysteine and oxidation of methionine, tryptophan and histidine were set as fixed and variable modifications, respectively. Maximally one missed cleavage was allowed. Additional information (e.g. peptide sequence, charge state of each peptide) will

be provided in supplementary data (Table S2 (leaf proteins), Table S3 (root proteins).

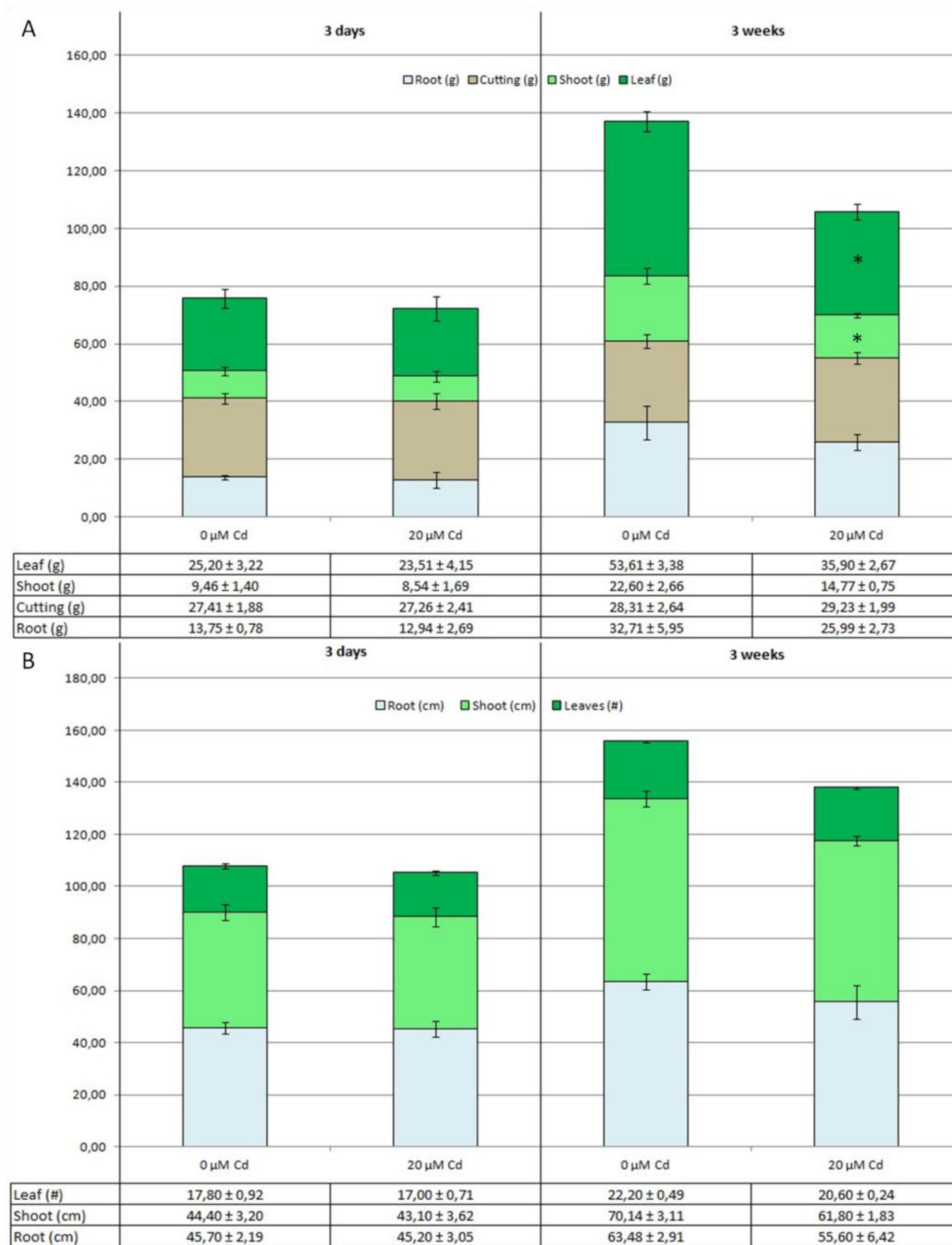
Resulting peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm described by Keller *et al.* (2002) within Scaffold version 2_05_02 (Proteome Software Inc., Portland, OR). Protein identifications were accepted if they could be established at greater than 99.9% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm within Scaffold according to Nesvizhskii *et al.* (2003). To assign a function, the accepted protein identifications were searched for homology against NCBI non-redundant database. Outcome of both search engines was visualized and validated with Scaffold v.3.6.1 (Proteome Software), with a final peptide and protein probability of 95% and 100%, respectively. For additional information concerning the identification we refer to the supplementary data (Table S2 (leaf proteins), Table S3 (root proteins)).

4.3.3 Results and discussion

4.3.3.1 General effects

After 3 days of Cd exposure, poplar cuttings did not show any visible toxicity symptoms. However after 21 days of exposure, light chlorosis was present on the poplar leaves. Only few cuttings showed necrotic spots near the main leaf vein. As described by Kieffer *et al.* (2008) these symptoms were restricted to young leaves which were expanding at the start of the exposure. Expanded leaves at the start of the exposure did not show any toxicity symptoms. Growth of poplar cuttings was not affected after 3 days of Cd exposure. However, after 21 days growth inhibition, based on the fresh weight, was present for cuttings exposed to Cd. This growth inhibition displayed at leaves, shoots and roots but significant differences were only present for leaf and shoot weight (Figure 4.3.2). Considering the number of leaves, the length of the shoot and the length of the root, a similar pattern was present; no inhibition was observed after 3 days of exposure while growth reduction was present after 21 days of Cd exposure. This growth reduction, although not significant, was present on all parameters, however most reduction was present on shoot length. Fagioni and Zolla (2009a) reported that leaves of Cd-exposed plants were about 60%

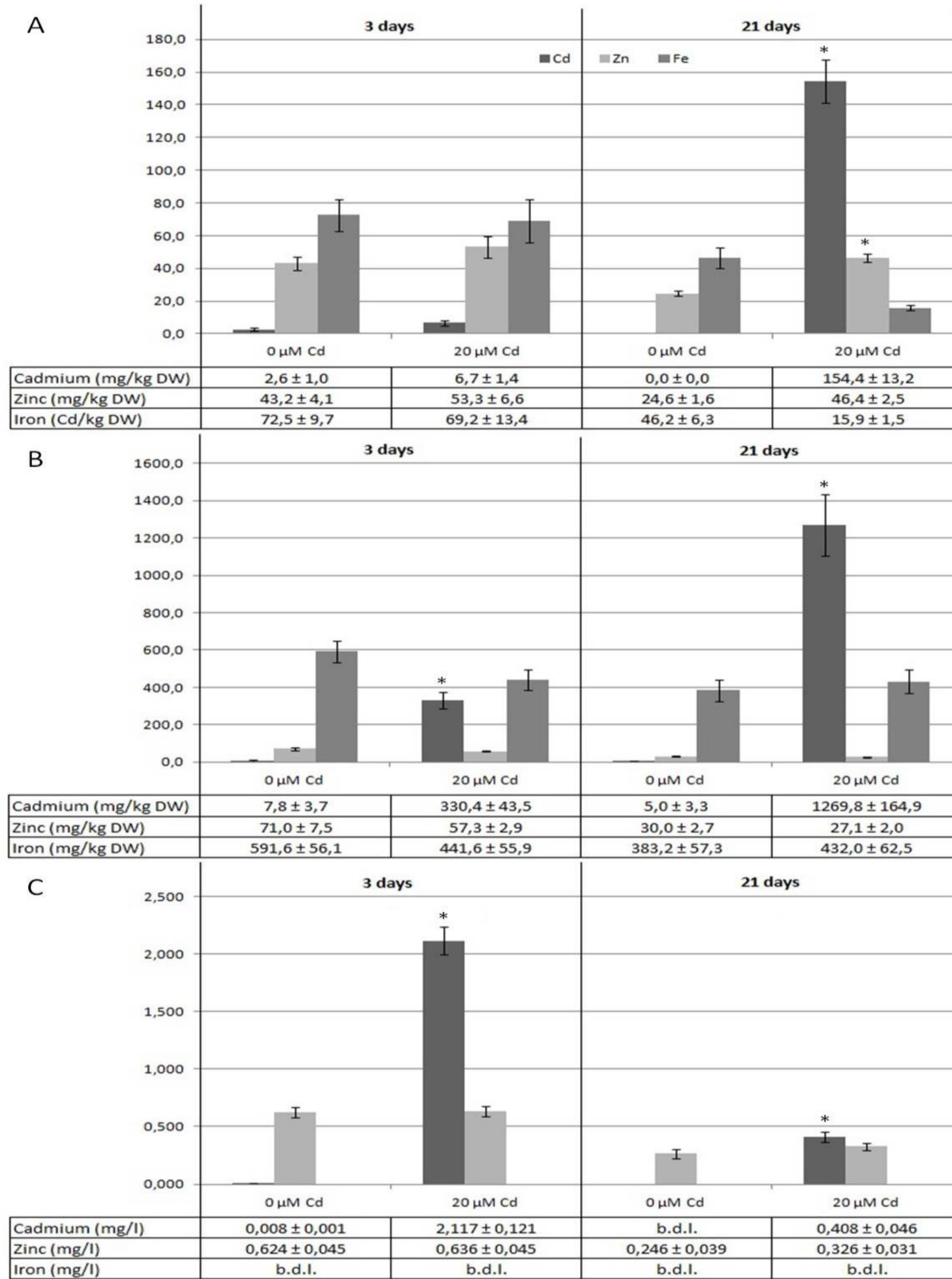
smaller than those of controls of the same age. Although Fagioni and Zolla (2009a) worked on *Arabidopsis*, their statement was postulated to be valid for all plant species. However, based on the growth reduction of leaves described in this experiment, we can not confirm this statement. Only a 33% decrease on leaf fresh weight and a 7% decrease on leaf number was present on poplar leaves after 21 days of Cd exposure. In order to provide all poplar plants with the same starting material, cuttings with the same weight were chosen, therefore no significant differences were present between the cutting weights.
Accumulation of Cd, Zn and Fe was measured after 3 days and 21 days of exposure (Figure 4.3.3). After 3 days of exposure, the content of these metals in the leaves was not significantly altered. In roots however, a significant accumulation of Cd was already present after 3 days of exposure, which is accompanied by a decreasing trend in Fe content. After 21 days of exposure, Cd content in leaves increased drastically. As described in roots after 3 days of exposure, this was accompanied by a decreasing trend in Fe content. Moreover, Zn content significantly increased in leaves after 21 days of exposure. In roots, Cd content also increased drastically after 21 days of exposure. No effect was present on Zn and Fe content. As depicted in figure 4.3.3C one might think that the Cd content in the hydroponic solution decreased after 21 days. However, this decrease is due to the fact that the hydroponic samples for determining the metal content after 3 days of exposure, were taken 3 days after solution refreshment, whereas the hydroponic samples for determining the metal content after 21 days of exposure were taken 7 days after solution refreshment. This indicates that the Cd content in the solution decreased within one refreshment cycle but never droped to zero, providing a constant supply of Cd during the experiment. Finally, the total Cd concentrations in leaves and roots (Figure 4.3.3A and 4.3.3B) are in line with those described when applying 20 µM $CdSO_4$ to hydroponically grown *P. tremula* L. cuttings (Kieffer *et al.*, 2008; 2009a).



	3 days 0 µM Cd	3 days 20 µM Cd	3 weeks 0 µM Cd	3 weeks 20 µM Cd
Leaf (g)	25,20 ± 3,22	23,51 ± 4,15	53,61 ± 3,38	35,90 ± 2,67
Shoot (g)	9,46 ± 1,40	8,54 ± 1,69	22,60 ± 2,66	14,77 ± 0,75
Cutting (g)	27,41 ± 1,88	27,26 ± 2,41	28,31 ± 2,64	29,23 ± 1,99
Root (g)	13,75 ± 0,78	12,94 ± 2,69	32,71 ± 5,95	25,99 ± 2,73

	3 days 0 µM Cd	3 days 20 µM Cd	3 weeks 0 µM Cd	3 weeks 20 µM Cd
Leaf (#)	17,80 ± 0,92	17,00 ± 0,71	22,20 ± 0,49	20,60 ± 0,24
Shoot (cm)	44,40 ± 3,20	43,10 ± 3,62	70,14 ± 3,11	61,80 ± 1,83
Root (cm)	45,70 ± 2,19	45,20 ± 3,05	63,48 ± 2,91	55,60 ± 6,42

Figure 4.3.2: Effects of Cd on (A) weight of leaves, shoots and roots and on (B) shoot length, root length and number of leaves. Data presented as average ± standard error. Asterisks present significant differences (2 way ANOVA, $p<0.05$) between control and samples of exposed cutting of the same exposure period.



A

	3 days, 0 µM Cd	3 days, 20 µM Cd	21 days, 0 µM Cd	21 days, 20 µM Cd
Cadmium (mg/kg DW)	2,6 ± 1,0	6,7 ± 1,4	0,0 ± 0,0	154,4 ± 13,2
Zinc (mg/kg DW)	43,2 ± 4,1	53,3 ± 6,6	24,6 ± 1,6	46,4 ± 2,5
Iron (Cd/kg DW)	72,5 ± 9,7	69,2 ± 13,4	46,2 ± 6,3	15,9 ± 1,5

B

	3 days, 0 µM Cd	3 days, 20 µM Cd	21 days, 0 µM Cd	21 days, 20 µM Cd
Cadmium (mg/kg DW)	7,8 ± 3,7	330,4 ± 43,5	5,0 ± 3,3	1269,8 ± 164,9
Zinc (mg/kg DW)	71,0 ± 7,5	57,3 ± 2,9	30,0 ± 2,7	27,1 ± 2,0
Iron (mg/kg DW)	591,6 ± 56,1	441,6 ± 55,9	383,2 ± 57,3	432,0 ± 62,5

C

	3 days, 0 µM Cd	3 days, 20 µM Cd	21 days, 0 µM Cd	21 days, 20 µM Cd
Cadmium (mg/l)	0,008 ± 0,001	2,117 ± 0,121	b.d.l.	0,408 ± 0,046
Zinc (mg/l)	0,624 ± 0,045	0,636 ± 0,045	0,246 ± 0,039	0,326 ± 0,031
Iron (mg/l)	b.d.l.	b.d.l.	b.d.l.	b.d.l.

Figure 4.3.3: Metal concentrations in (A) poplar leaves, (B) poplar roots and (C) hydroponic solution. Data presented as average ± standard error, b.d.l. = below detection limit. Asterisks present significant differences (2 way ANOVA, $p<0.05$) between control and samples of exposed cuttings of the same exposure period.

4.3.3.2 Effects of short and long term exposure to Cd on poplar

All identified proteins with a fold change > |1.5| (ANOVA < 0.05) were manually classified into functional groups. As discussed in chapter 4.2, the energy metabolism comprises light reactions of photosynthesis and mitochondrial electron transport chain. The carbohydrate metabolism comprises proteins involved in (1) "CO_2-fixation"; comprising carbon reactions of photosynthesis, (2) "Catabolism"; comprising glycolysis/gluconeogenesis, TCA cycle, pyruvate metabolism and pentose phosphate pathway and (3) "Metabolism of C-based molecules"; comprising all other carbohydrate metabolism subgroups, as listed in the KEGG PATHWAY database (http://www.genome.jp/kegg/pathway.html). This functional classification was chosen to get a better insight into the energy flow during Cd exposure. Proteins to which no clear function could be assigned, were grouped into 'others'.

4.3.3.2.1 Effects on poplar leaves at proteome level

Approximately 1500 spots could be detected on each gel. Of these, 799 spots could be matched on 90% of all gels and were included in the statistical analysis. A two-way ANOVA (condition 1: time, condition 2: exposure) was performed on the dataset identifying 198 spots with significant differential expression ($p<0.05$). Of these, 89 spots had an absolute variation of at least 1.5-fold between control and exposed samples. A PCA analysis showed a clear separation of the Cd-exposed group after 21 days along the first axis (Figure 4.3.4). No separation can be made between control and Cd-exposed groups after 3 days of exposure. The control group after 21 days is only slightly separated from the control and Cd-exposed group after 3 days of exposure, indicating only a moderate time effect during the experiment.

A hierarchical classification confirms the clustering of control and Cd-exposed groups after 3 days of exposure (Figure 4.3.5). As indicated by the PCA analysis, control group after 21 days clusters more closely to the groups after 3 days of exposure and the Cd-exposed group after 21 days of exposure is separated from the others. Moreover, a high up-regulation is present after 21 days of Cd exposure.

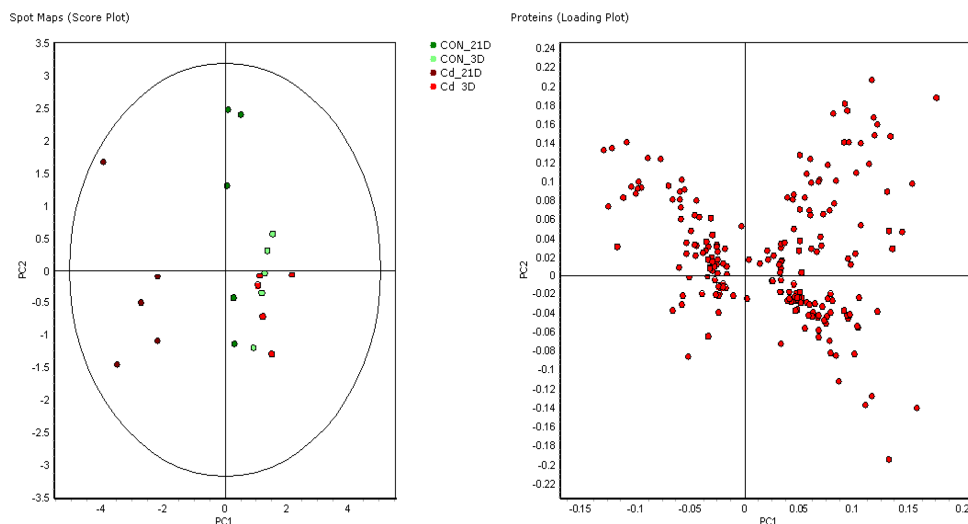


Figure 4.3.4: PCA analysis of 198 differentially abundant leaf proteins ($p<0.05$; two-way ANOVA). The left panel shows the distribution of spot maps regarding the two principal components (PC1 and PC2 explaining a cumulated 77.3% of all variation). The right panel shows the distribution of the proteins.

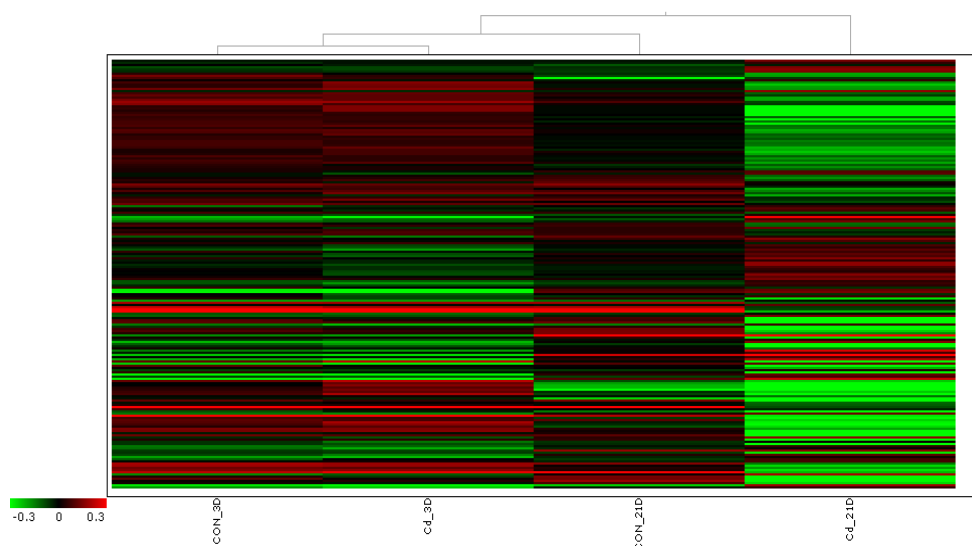


Figure 4.3.5: Hierarchical clustering of 198 differentially abundant leaf proteins ($p<0.05$, two-way ANOVA). Controls (day 3 + 21) and Cd-exposed groups after 3 days of exposure are grouped together. Cadmium-exposed group after 21 days of exposure is separated from the others.

Table 4.3.2: List of identified proteins extracted from hydroponically grown leaves after 3 and 21 days of Cd exposure. CON: control, CAD: cadmium.

Master No.	GI number	Protein name	Appearance	1-ANOVA	Av. Ratio CON 3 - CAD 3	Av. Ratio CON 21 - CAD 21
Energy metabolism						
318	133712068	ATP synthase cf1 beta subunit	30 (30)	2,3E-03	1,05	**-1,55**
321	133712068	ATP synthase cf1 beta subunit	30 (30)	2,0E-04	-1,06	**-1,68**
684	222866808	Peptidyl-prolyl cis-trans isomerase cyp38	30 (30)	3,5E-02	-1,11	**-1,60**
697	222866808	Peptidyl-prolyl cis-trans isomerase cyp38	30 (30)	3,7E-02	-1,10	**-1,56**
963	118485261	ATP synthase d	30 (30)	1,1E-04	-1,03	**-2,06**
	222864320					
981	222853091	Oxygen-evolving enhancer protein chloroplast	30 (30)	2,5E-05	-1,05	**-3,87**
1007	222853091	Oxygen-evolving enhancer protein chloroplast	30 (30)	1,6E-03	-1,01	**-3,33**
1012	222853091	Oxygen-evolving enhancer protein chloroplast	30 (30)	1,3E-03	-1,21	**-3,40**
1030	222856683	Oxygen-evolving enhancer protein chloroplast	30 (30)	1,2E-02	-1,11	**-3,00**
1036	222856683	Oxygen-evolving enhancer protein chloroplast	30 (30)	1,2E-03	-1,08	**-2,38**
1070	222856683	Oxygen-evolving enhancer protein chloroplast	30 (30)	1,9E-02	-1,18	**-2,70**
1073	222856683	Oxygen-evolving enhancer protein chloroplast	30 (30)	9,0E-03	-1,10	**-1,92**

Table 4.3.2: List of identified proteins extracted from hydroponically grown leaves after 3 and 21 days of Cd exposure CON: control, CAD: cadmium (Continued).

Master No.	GI number	Protein name	Appearance	1-ANOVA	Av. Ratio CON 3 - CAD 3	Av. Ratio CON 21 - CAD 21
Energy metabolism						
1166	224114357	Light-harvesting complex II protein lhcb1	30 (30)	5,3E-03	1,10	**-1,64**
	222856376					
	222857174					
1253	222842584	Oxygen-evolving enhancer protein chloroplastic-like	30 (30)	1,2E-03	-1,09	**-2,26**
1256	222861991	Light-harvesting complex i chlorophyll a b binding protein 3	27 (30)	2,2E-04	1,27	**-1,81**
1259	222861991	Light-harvesting complex i chlorophyll a b binding protein 3	27 (30)	2,5E-04	1,22	**-2,48**
1261	222871777	Light-harvesting complex ii protein lhcb3	30 (30)	6,2E-05	-1,00	**-1,66**
	224117282					
1271	222842584	Oxygen-evolving enhancer protein chloroplastic-like	30 (30)	4,8E-03	1,01	**-2,22**
1277	222857019	Oxygen-evolving enhancer protein chloroplastic-like	30 (30)	7,6E-05	-1,11	**-3,17**
1297	222861991	Light-harvesting complex i chlorophyll a b binding protein 3	30 (30)	4,5E-03	-1,08	**-1,97**
1387	222853091	Oxygen-evolving enhancer protein chloroplast	30 (30)	6,8E-04	-1,02	**-2,04**
1407	222862443	ATP synthase delta chain	30 (30)	1,1E-04	-1,06	**-1,68**

Table 4.3.2: List of identified proteins extracted from hydroponically grown leaves after 3 and 21 days of Cd exposure CON: control, CAD: cadmium (Continued).

Master No.	GI number	Protein name	Appearance	1-ANOVA	Av. Ratio CON 3 - CAD 3	Av. Ratio CON 21 - CAD 21
Protein metabolism						
152	222847569	Heat shock 70 kda mitochondrial-like	30 (30)	2,5E-02	-1,04	**1,57**
155	222850363	Heat shock 70 kda mitochondrial-like	30 (30)	4,6E-04	-1,03	**1,66**
570	222852553	Glutamine synthetase	30 (30)	4,4E-02	-1,04	**-1,74**
	224107259					
Carbohydrate metabolism						
385	158513644	Ribulose-1,5-bisphosphate carboxylase oxygenase large subunit	30 (30)	1,4E-02	1,17	**-1,73**
387	158513644	Ribulose-1,5-bisphosphate carboxylase oxygenase large subunit	27 (30)	3,9E-02	1,13	**-1,57**
394	158513644	Ribulose-1,5-bisphosphate carboxylase oxygenase large subunit	30 (30)	8,9E-03	1,06	**-1,57**
651	118486739	Ribulose bisphosphate carboxylase oxygenase activase chloroplast	30 (30)	7,8E-03	-1,04	**-1,52**
	222851930					
1183	222849388	Triosephosphate isomerase	30 (30)	9,1E-03	1,26	**-2,16**
	118487168					
	222870542					
	222870543					

Table 4.3.2: List of identified proteins extracted from hydroponically grown leaves after 3 and 21 days of Cd exposure CON: control, CAD: cadmium (Continued).

Master No.	GI number	Protein name	Appearance	1-ANOVA	Av. Ratio CON 3 - CAD 3	Av. Ratio CON 21 - CAD 21
Stress metabolism						
969	222833311	Germin-like protein	30 (30)	3,9E-06	-1,09	**-1,94**
1137	222849914	Ascorbate peroxidase	30 (30)	1,5E-03	-1,23	**-3,30**
1197	222867481	Ascorbate peroxidase	30 (30)	1,0E-04	-1,19	**-4,03**
1388	222856014 222853989	*S*-formylglutathione hydrolase	30 (30)	3,5E-05	-1,02	**-1,85**
Nucleotide metabolism						
488	222852016	High chlorophyll fluorescent 109 protein	30 (30)	1,9E-03	1,03	**-1,75**
Others						
1287	118486083	Protein	30 (30)	9,1E-05	-1,01	**-2,28**
1590	222837354	Pentatricopeptide repeat-containing protein	27 (30)	2,9E-03	-1,18	**2,83**
229	222852729 335345645	Polyphenol oxidase	30 (30)	3,7E-05	-1,09	**1,73**

Master No represents the spot number on the master gel. GI number represents the corresponding accession number in the NCBInr database. Protein name obtained via Blast2Go software by blasting against the NCBInr database. Appearance indicates the number of gels in which the spot appeares, the total gel number is given between brackets. 1-ANOVA represents the p-value with $\alpha = 0,05$. Average ratio of the protein abundance is given for 3 and 21 days of exposure, positive values are gives as such, while negative values are given according to the following formula: given value = -1/average ratio.

Differentially abundant proteins with an absolute variation of at least 1.5-fold ($p<0.05$) were picked and submitted for identification. Of those, 38 proteins could be identified unambiguously (Table 4.3.2) and will be discussed hereafter. In the subsequent discussion, proteins will be referred to by their protein name, as inferred by searched the accepted protein identifications for homology against NCBI non-redundant database.

Table 4.3.3: Functional grouping of identified proteins extracted from hydroponically grown leaves after 3 and 21 days of Cd exposure. Absolute numbers (#) and percentages (%) are given. Percentages are calculated per conditional group.

	3 days				21 days			
	UP		DOWN		UP		DOWN	
	#	%	#	%	#	%	#	%
Energy metabolism	-		-		-		22	57.9
Protein metabolism	-		-		2	5.3	1	2.6
Carbohydrate metabolism	-		-		-		5	13.2
Stress metabolism	-		-		-		4	10.5
Nitrogen metabolism	-		-		-			
Nucleotide metabolism	-		-		-		1	2.6
Others	-		-		2	5.3	1	2.6
Total	**0**				**38**			

To map the Cd effect, Cd samples were compared to control samples after 3 days of exposure and after 21 days of exposure. After 3 days of Cd exposure, no proteins differed in abundance, indicating the occurrence of a ‘delayed effect’ in poplar leaves. In contrast, after 21 days of exposure, 38 proteins differed in abundance of which four were up-regulated and 34 were down-regulated. Upon 21 days of Cd exposure, six classes were addressed (Table 4.3.3): energy metabolism (22), carbohydrate metabolism (5), stress metabolism (4), protein metabolism (3), nucleotide metabolism (1) and others (3).

After 21 days of Cd exposure, many proteins involved in energy production were down-regulated. As frequently described in leaves of Cd-exposed plants, oxygen-evolving enhancer proteins, light harvesting complex proteins and ATP synthases were affected; however the previously reported responses of these proteins were not unambiguous since both, up- and down-regulations were described (Alvarez *et al.*, 2009; Fagioni and Zolla, 2009a; Kieffer *et al.*, 2009a,

2009b; Durand *et al.*, 2010; Rodriguez-Celma *et al.*, 2010; Zhoa *et al.*, 2011). Peptidyl-prolyl cis-trans isomerase, which is required for the assembly and stabilization of PSII (Fu *et al.*, 2007), was down-regulated as well. A closer look into the carbohydrate metabolism reveals a down-regulation in CO_2 fixation by affecting RuBisCO large subunits and a down-regulation of chloroplastic triosephosphate isomerase. Upon Cd exposure, triosephosphate isomerase was previously reported to be both up- and down-regulated in leaves (Kieffer *et al.*, 2008, 2009a, 2009b; Fagioni and Zolla, 2009a; Durand *et al.*, 2010). Next to energy metabolism and carbohydrate fixation, a clear down-regulation of stress metabolism is present. Germin-like protein is a known stress defence protein and was previously described to be differentially abundant in plants exposed to Cd (Alvarez *et al.*, 2009). In contrast to leaves, germin-like protein was down-regulated in *Brassica juncea* roots upon Cd stress (Alvarez *et al.*, 2009). Ascorbate peroxidases are known to reduce H_2O_2 (Shigeoka *et al.*, 2002), a reactive oxygen species induced by Cd exposure (Keunen *et al.*, 2011). In the past, ascorbate peroxidases were described to be down-regulated in *Brassica juncea* roots upon Cd exposure (Alvarez *et al.*, 2009). *S*-formylglutathione hydrolase is thought to play a role in formaldehyde detoxification (Haslam *et al.*, 2002). Formaldehyde is a reactive compound derived from photo-oxidation of atmospheric hydrocarbons. Formaldehyde spontaneously reacts with glutathione to produce *S*-hydromethylglutathione (Barber and Donohue, 1998). Next, glutathione dependent formaldehyde dehydrogenase enzymes (GSH-FDH) oxidize *S*-hydroxymethylglutathione to *S*-formylglutathione with concomitant reduction of NAD (Barber and Donohue, 1998). NADH production is relevant upon Cd exposure since it is important in ROS detoxification. Finally, *S*-formylglutathione is converted to GSH and formate by the action of *S*-formylglutathione hydrolase (Barber and Donohue, 1998). By releasing GSH, this component can act as a Cd chelator (Alvarez *et al.*, 2009). In this way, *S*-formylglutathione hydrolase can be responsible for detoxification of Cd. Upon stress, protein metabolism is often up-regulated to provide specific proteins that can lower stress effects. On the other hand, up-regulation of proteins involved in protein folding is probably due to the strong reducing effect of Cd, resulting in the destabilization of proteins. In this context, heat shock protein 70 kDa is up-regulated in leaves of Cd-exposed poplar. Glutamine synthetase however was

down-regulated upon 21 days of Cd exposure. In previous reports, glutamine synthetase was mostly described to be up-regulated upon Cd exposure (Kieffer *et al.*, 2008, 2009a, 2009b; Semane, 2010). However, Kieffer *et al.* (2009b) also reported a down-regulation of glutamine synthetase in leaves of Cd-exposed poplar. Since glutamine is an important precursor of GSH, down-regulation of glutamine synthetase is somewhat surprising. Finally, a high chlorophyll fluorescent 109 protein was down-regulated after 21 days of Cd exposure. High chlorophyll fluorescent 109 protein is believed to be a translation release factor (Meurer *et al.*, 2002). Meurer *et al.* (1996) indicated that the *hcf109* mutant specifically affects the expression of several plastome-encoded photosystem I and II genes. Phenotypically, the *hcf109* mutant was classified as a pleiotropic mutant whose photosynthetic electron transport chain is almost totally inactive due to defects in photosystems I and II and the plastid NAD(P)H dehydrogenase complex (Meurer *et al.*, 1996). These data indicate that HCF109 is somehow important for maintaining photosynthesis. Therefore the observed decrease in protein abundance is somewhat unexpected since leaves of Cd-exposed plants are known to encounter damage to the photosynthesis machinery (Bi *et al.*, 2009).

4.3.3.2.2 Effects on poplar roots at proteome level

For roots, approximately 2500 spots could be detected on each gel of which 1433 could be matched on 90% of all the gels and were included in the statistical analysis. Significant differential expression was present for 601 spots (two way ANOVA; condition 1: time, condition 2: exposure; $p<0.05$) between control and exposed samples. However, only 148 proteins had an absolute variation of at least 1.5-fold between control and exposed samples and were subjected to identification. Of those, only 33 were identified unambiguously. PCA analysis showed a clustering of control and Cd-exposed groups after 3 days of exposure (Figure 4.3.6). In contrast to leaves, the control group after 21 days is clearly separated from the 3 days exposed groups, which might indicate a time effect on the root proteome. A clear separation of the Cd group after 21 days of exposure is present along the first and second axis.

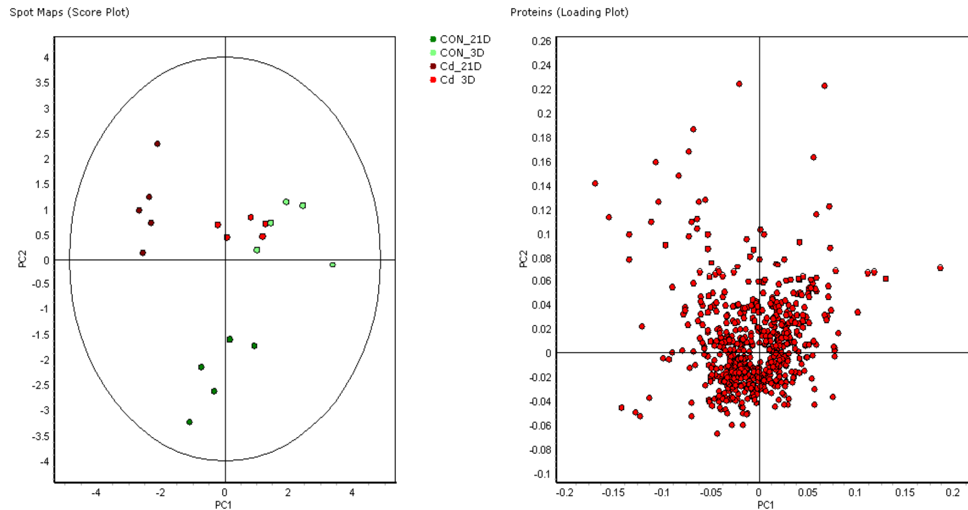


Figure 4.3.6: PCA analysis of 601 differentially abundant root proteins ($p<0.05$; two-way ANOVA). The left panel shows the distribution of spot maps regarding the two principal components (PC1 and PC2 explaining a cumulated 54.7% of all variation). The right panel shows the distribution of the proteins.

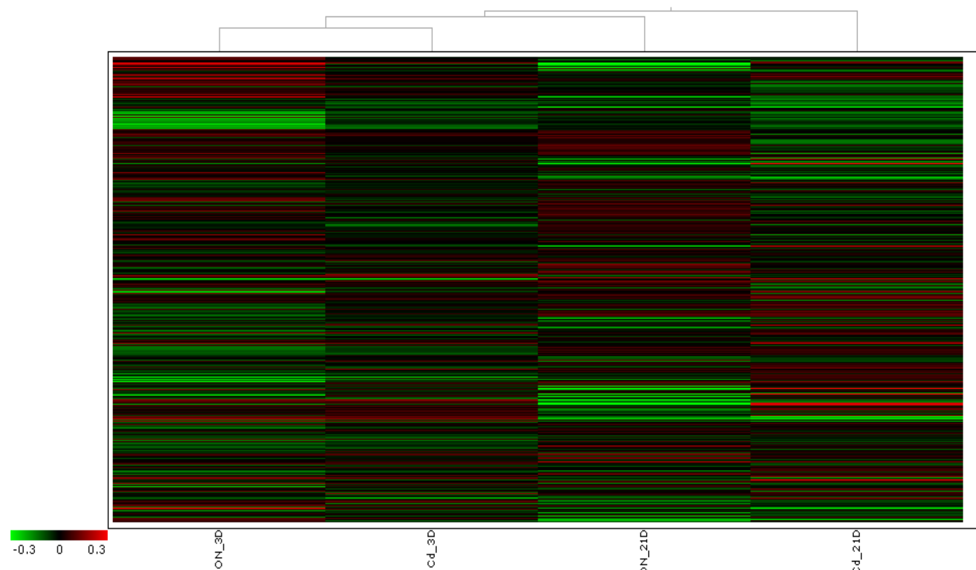


Figure 4.3.7: Hierarchical clustering of 601 differentially abundant root proteins ($p<0.05$, two-way ANOVA). Controls (day 3 + 21) and Cd-exposed groups after 3 days of exposure are grouped together. Cadmium-exposed group after 21 days of exposure is separated from the others.

Table 4.3.4: List of identified proteins extracted from hydroponically grown roots after 3 and 21 days of Cd exposure. CON: control, CAD: cadmium.

Master No.	GI number	Protein name	Appearance	1-ANOVA	Av. Ratio CON 3 - CAD 3	Av. Ratio CON 21 - CAD 21
Protein metabolism						
599	222837523	Lysyl-trna synthetase	30 (30)	4,8E-06	1,06	**2,7**
	224143682					
1413	224079530	Glutamine synthetase	30 (30)	3,4E-05	-1,01	**-1,65**
734	222841415	tcp-1 cpn60 chaperonin family protein	30 (30)	9,3E-03	**-1,52**	-1,14
	222844875					
1389	224106760	Isovaleryl-CoA dehydrogenase 1	30 (30)	1,0E-02	1,07	**1,78**
1925	222833477	Thiol protease aleurain	30 (30)	5,2E-04	1,15	**1,8**
2073	222862963	Kunitz trypsin inhibitor 3	30 (30)	1,6E-02	**2,24**	-1,09
	242346649					
	242346685					
2078	222854239	Proteasome subunit alpha	27 (30)	2,2E-03	1,32	**1,88**
2466	222862963	Kunitz trypsin inhibitor 3	30 (30)	6,7E-04	1,13	**1,62**
	242346649					
	242346685					
Carbohydrate metabolism						
191	158513644	Ribulose-1,5-bisphosphate carboxylase oxygenase large subunit	27 (30)	4,8E-03	**-2,11**	**2,39**
2017	118485535	Triosephosphate isomerase	30 (30)	8,8E-03	-1,01	**-1,59**
	224106477					
1050	222845250	6-phosphogluconate dehydrogenase	30 (30)	7,6E-06	-1,14	**-1,5**
1355	224101335	Phosphoglycerate kinase	30 (30)	2,0E-02	1,21	**-1,63**
1363	222861274	Formate dehydrogenase	27 (30)	3,2E-05	1,07	**2,12**

Table 4.3.4: List of identified proteins extracted from hydroponically grown roots after 3 and 21 days of Cd exposure CON: control, CAD: cadmium (Continued).

Master No.	GI number	Protein name	Appearance	1-ANOVA	Av. Ratio CON 3 - CAD 3	Av. Ratio CON 21 - CAD 21
Stress metabolism						
2328	118485257	Glutathione peroxidase	30 (30)	2,8E-02	**1,62**	**2,37**
	222841015					
1153	118486403	DNA repair protein rad23-1	27 (30)	1,9E-02	1,06	**1,58**
	222849705					
2195	118486098	Predicted tau class glutathione transferase GSTU33	30 (30)	3,2E-05	1,43	**1,9**
	283135866					
2420	222851328	Major latex	30 (30)	1,7E-02	1,07	**-2,19**
	222846794					
2426	222865076	Universal stress protein	30 (30)	7,0E-03	1,21	**3,27**
2508	222847956	PR-10 protein	30 (30)	6,2E-04	-1,30	**-1,63**
	222838565					
Cell - Structure, growth and maintenance						
1291	118481267	Actin	30 (30)	2,2E-02	-1,49	**1,53**
	222845968					
	222860713					
	222874629					
1625	222843751	Phenylcoumaran benzylic ether reductase	27 (30)	3,8E-02	**1,58**	-1,24
	3114903					
	49455471					

Table 4.3.4: List of identified proteins extracted from hydroponically grown roots after 3 and 21 days of Cd exposure CON: control, CAD: cadmium (Continued).

Master No.	GI number	Protein name	Appearance	1-ANOVA	Av. Ratio CON 3 - CAD 3	Av. Ratio CON 21 - CAD 21
Nucleotide metabolism - Transcription/Translation						
2403	222854376	Eukaryotic translation initiation factor 5a3	30 (30)	4,0E-02	-1,03	**1,64**
Others						
1328	118486083 118487168 222870543	Protein - SGNH plant lipase like	30 (30)	1,4E-02	**1,91**	1,18
1344	222849705	Cinnamyl alcohol dehydrogenase-like protein	30 (30)	9,9E-06	1,13	**2,04**
1359	222831876	Cinnamyl alcohol dehydrogenase-like protein	27 (30)	2,4E-07	1,22	**6,65**
1496	222871716	Hyoscyamine 6-dioxygenase-like	30 (30)	1,8E-03	-1,29	**-1,51**
2105	222859762	Flavoprotein wrba-like	27 (30)	7,5E-03	1,23	**1,96**
2135	118486083 118487168 222870543	Protein - SGNH plant lipase like	30 (30)	2,2E-02	**3,03**	1,11
2225	222860018	Quinone reductase family protein	30 (30)	1,7E-02	1,46	**1,89**
2292	222864803	Gcn5-related n-acetyltransferase-like protein	27 (30)	4,9E-04	1,38	**3,19**
2485	222838565	Protein	30 (30)	1,9E-02	-1,00	**1,95**

Master No represents the spot number on the master gel. GI number represents the corresponding accession number in the NCBInr database. Protein name obtained via Blast2Go software by blasting against the NCBInr database. Appearance indicates the number of gels in which the spot appeares, the total gel number is given between brackets. 1-ANOVA represents the p-value with $\alpha = 0,05$. Average ratio of the protein abundance is given for 3 and 21 days of exposure, positive values are gives as such, while negative values are given according to the following formula: given value = -1/average ratio.

A hierarchical classification of these 601 differentially abundant proteins confirms the clustering of control and Cd-exposed groups after 3 days of exposure (Figure 4.3.7). The control group after 21 days clusters more closely to the groups after 3 days of exposure than the Cd-exposed group after 21 days of exposure. The high up-regulation in leaves after 21 days of Cd exposure, is not present in the root samples.

In order to map the Cd effect, a comparison between control and Cd-exposed samples was made upon 3 and 21 days of exposure. After 3 days of exposure, only seven root proteins differed in abundance, indicating only a minor stress response after short term exposure. Of these, four proteins were down-regulated and three up-regulated. Differentially abundant proteins could be grouped into five functional classes: Protein metabolism (2), carbohydrate metabolism (1), stress metabolism (1), cell – structure, growth and maintenance (1) and others (2). Kunitz trypsin inhibitor 3, involved in protein degradation, is up-regulated whereas a tcp-1 cpn60 chaperonin family protein, involved in protein assembly and folding was down-regulated. Up-regulation of proteins involved in protein degradation can be seen as an attempt to re-allocate amino acids to respond accurately to Cd stress. Since the presence of Cd often results in destabilization of proteins (Ashan *et al.*, 2009), the down-regulation of tcp-1 cpn60 chaperonin family protein is somewhat unexpected. Of proteins involved in carbohydrate metabolism, only RuBisCO was down-regulated. The presence of RuBisCO in roots seems erroneous at first, however it is known that roots tend to differentiate chloroplasts upon exposure to light (Flores *et al.*, 1993). It should be mentioned that despite major efforts to prevent light to enter the hydroponic solution, a limited amount was still able to reach the roots through the holes of the water refill tube and the air tube (Figure 4.3.1), what could explain the presence of RuBisCO found in poplar roots in this experiment. As an attempt to detoxify Cd, glutathione peroxidase was up-regulated. Phenylcoumaran benzylic ether reductase (PCBER), a prominent poplar xylem protein involved in phenylpropanoid biosynthesis, is up-regulated as well. PCBER is preferentially produced in the secondary xylem of stems and roots and is associated with the active growth period (Vander Mijnsbrugge *et al.*, 2000a),

however its biological function remains unclear (Vander Mijnsbrugge *et al.*, 2000b).

After 21 days of Cd exposure 26 proteins differed in abundance (Table 4.3.4). Functional analysis of the differentially abundant proteins shows that most proteins are involved in protein metabolism (6) and stress metabolism (6), followed by carbohydrate metabolism (5), cell - structure, growth and maintenance (1) and nucleotide metabolism (1) and finally seven not classified proteins (Table 4.3.5).

Table 4.3.5: Functional grouping of identified proteins extracted from hydroponically grown roots after 3 and 21 days of Cd exposure. Absolute numbers (#) and percentages (%) are given. Percentages are calculated per conditional group.

	3 days				21 days			
	UP		DOWN		UP		DOWN	
	#	%	#	%	#	%	#	%
Protein metabolism	1	14.3	1	14.3	5	20.0	1	4.0
Carbohydrate metabolism			1	14.3	2	8.0	3	12.0
Stress metabolism	1	14.3			4	16.0	2	8.0
Cell - Structure, growth and maintenance	1	14.3			1	4.0		
Nucleotide metabolism					1	4.0		
Others			2	28.6	6	24.0	1	4.0
Total	**7**				**26**			

Protein metabolism is mainly up-regulated. Among the up-regulated protein belong a lysyl-tRNA synthetase which inserts lysine into proteins (Freist and Gauss, 1995) and several degradation proteins: isovaleryl-CoA dehydrogenase 1, proteasome subunit alpha, a Kunitz trypsin inhibitor 3 and a thiol protease aleurain which is thought to function in nitrogen mobilization from protein reserves (Hensel *et al.*, 1993). Glutamine synthetase was the only down-regulated protein. In contrast, glutamine synthetase was described to be up-regulated in roots of Cd-exposed *Arabidopsis thaliana* (Semane *et al.*, 2010). Glutamine synthetase is essential for nitrogen metabolism since it catalyzes the condensation of glutamate and ammonia to glutamine. Glutamine is an important constituent of GSH and therefore down-regulation of glutamine synthetase is unexpected upon Cd exposure. However down-regulation was also described by Kieffer *et al.* (2009b). Concerning the carbohydrate metabolism,

two proteins of the glycolysis are down-regulated upon 21 days of Cd exposure. Triosephosphate isomerase converts the triose phosphates – derived from sucrose in the cytosol or starch from the chloroplast – into glyceraldehyde-3-phosphate, confirming the down-regulation reported by Kieffer *et al.* (2009b) and Rodriguez-Celma *et al.* (2010) in poplar and tomato roots respectively.

Phosphoglycerate kinase is also down-regulated and is one of the enzymes involved in the energy conserving phase of glycolysis. In this phase, glyceraldehyde-3-phosphate is converted further to pyruvate (Taiz and Zeiger, 2002). Previously, in Cd-exposed roots, phosphoglycerate kinase has been reported to be down-regulated (Alvarez *et al.*, 2009; Rodriguez-Celma *et al.*, 2010). Furthermore 6-phosphogluconate dehydrogenase was down-regulated in poplar roots after 21 days of Cd exposure. 6-phosphogluconate dehydrogenase is involved in the pentose phosphate pathway where it converts 6-phosphogluconate to ribulose-5-phosphate, hereby releasing CO_2.

Formate dehydrogenase was up-regulated in poplar roots, confirming the described up-regulation in Cd-exposed *Brassica juncea* roots (Alvarez *et al.*, 2009). Formate dehydrogenase catalyzes the oxidation of formate to CO_2 (Ferry, 1990). Upon this reaction, NAD^+ is reduced to NADH which can be used to counterbalance the negatively affected ROS balance, induced by Cd. After 3 days of Cd exposure, RuBisCO presence was detected in poplar roots despite all efforts to avoid light at root level. After 21 days of Cd exposure, even an up-regulation of RuBisCO was present.

Stress metabolism is up-regulated by increasing abundance in glutathione peroxidase, glutathione-S-transferase, DNA repair protein rad23-1 and a universal stress protein (USP). Glutathione peroxidase was also up-regulated after 3 days of Cd exposure, however 21 days of exposure further increased the differential expression in abundance. Glutathione-S-transferase is involved in antioxidant detoxification systems which control the cascades of uncontrolled oxidation and protect plant cells from oxidative damage by scavenging of ROS (Gill and Tuteja, 2010). DNA repair protein rad23-1 is known to be a negative regulator of multi-Ubiquitin (Ub) chain assembly (Ortolan *et al.*, 2000; Chen *et al.*, 2001). It is suggested that its interaction with Ub is important in DNA repair (Guzder *et al.*, 1995), stress response (Lambertson *et al.*, 1999) and cell cycle progression (Clarke *et al.*, 2001). Moreover, it has been proposed that it could

control protein stability by binding and preventing the expansion of nascent multi-Ub chain (Ortolan *et al.*, 2000). To our knowledge, it has not been reported previously to be affected by the presence of Cd. Universal stress proteins are found in bacteria, Archaea, fungi, flies and plants (Kvint *et al.*, 2003). Genetic evidence has shown that UspA mediates survival of cells starved for a wide variety of nutrients, exposed to toxic chemicals and exposed to osmotic stress or UV light damage (Kerk *et al.*, 2003). In addition, a role in oxidative stress responses has been reported as well (Nachin *et al.*, 2005). In contrast to the findings of Roth *et al.* (2006), major latex protein is down-regulated in poplar roots upon Cd exposure. The function of major latex proteins is still unknown but they have been associated with fruit and flower development and pathogen defence response (Lytle *et al.*, 2009). Since major latex protein are involved in stress defence, its down-regulation upon Cd exposure is unexpected. The down-regulation of PR-10, known to be expressed upon pathogen infection as well as abiotic stress such as salinity and drought (Dubos and Plomion, 2001), was somewhat unexpected since most reported PR proteins are up-regulated upon Cd exposure (Kieffer *et al.*, 2009b; Semane *et al.*, 2010; Rodriguez-Celma *et al.*, 2010). Actin was the only protein that was functionally classified to be involved in cell – structure, growth and maintenance. In correspondence to the findings of Alvarez *et al.* (2009, *Brassica juncea* roots) and Rodriguez-Celma *et al.* (2010, *Lycopersicon esculentum* Mill cv. Tres Cantos roots) it was up-regulated in poplar roots. Actin is one of the three major components of the cytoskeleton and is involved in intracellular transport and cellular division (Higaki *et al.*, 2007). Since actin is involved in nuclear export of RNAs and proteins (Zheng *et al.*, 2009), it might be necessary to allow a specific stress response. Finally, in poplar roots after 21 days of Cd exposure, eukaryotic translation initiation factor 5a3 was up-regulated. This protein is thought to be involved in programmed cell death (Hopkins *et al.*, 2008). Hereby, the up-regulation of translation initiation factor 5a3 might play a role in Cd-induced programmed cell death.

4.3.4 Conclusion

An exposure period of 3 days was too short to induce visible symptoms of toxicity. Moreover, growth reduction was not present in Cd-exposed cuttings compared to control ones (Figure 4.3.2). In leaves only a minor increasing trend in Cd content was present (Figure 4.3.3), while a significant increase in Cd content was present in Cd-exposed roots (Figure 4.3.3). The absence of a morphological Cd effect after 3 days of exposure was confirmed by proteomic analysis of leaves where no differentially expression was present at 3 days of Cd exposure. In roots a limited Cd effect on the proteome was present after 3 days of Cd exposure: 7 proteins differed in abundance and could be correlated to the high Cd content present in roots. These data suggest that an exposure time of 3 days was not sufficient to induce significant differences on leaf proteome of hydroponically grown *P. deltoides x (trichocarpa x deltoides)* cuttings, as it was described by Kieffer *et al.* (2008, 2009b). In addition, based on PCA analysis (Figure 4.3.4) and hierarchical clustering (Figure 4.3.5) no separation could be made between control and cadmium gels after 3 days of exposure. In roots, no clear up- or down-regulation in a specific pathway or metabolism was present. Although the meta-analysis of Cd-induced proteome changes (see section 4.2. 6.1.1.2) indicates that stress metabolism and carbohydrate metabolism are mainly addressed in short term exposed roots, in this study a more or less even distribution of differentially abundant proteins was present. In the large time scale study on poplar leaf and root proteome of Kieffer *et al.* (2009b) only 3 proteins differed in abundance upon Cd exposure. Data of Kieffer *et al.* (2009b) together with the data obtained in this study indicate that only a minor Cd effect is manifested in leaves and roots of hydroponically grown poplar cuttings after 3 days of exposure.

Exposing *P. deltoides x (trichocarpa x deltoides)* to Cd during 21 days, resulted in a decreasing trend in plant growth. Significant decreases were present on leaf and shoot mass (Figure 4.3.2). Compared to control cuttings, Cd content was significantly higher is leaves, shoots and roots of Cd-exposed cuttings (Figure 4.3.3). On the proteome level, a stronger effect of Cd was present than at 3 days exposure: in leaves 38 proteins differed in abundance, while in roots 25 proteins differed in abundance. The higher amount of differentially abundant proteins in leaves compared to roots is in line with the findings in the meta-

analysis of Cd-induced proteome effects in plants (see section 4.2.6.1.1). The general down-regulation of CO_2 fixation, chloroplast electron transport chain and ATP synthases in leaves, described in the meta-analysis data, is confirmed by our data. However, contrasting results were obtained for proteins involved in the stress metabolism. In our study stress metabolism was down-regulated in leaves whereas it was typically up-regulated considering the meta-analysis data. Some proteins were unexpectedly down-regulated in leaves of cuttings exposed to Cd for 21 days. The unexpected down-regulation of glutamine synthetase and a high chlorophyll fluorescent 109 protein could be seen a result of a shift in stress avoidance strategies. This means that it could be that those proteins were initially addressed but by shifting into another stress avoidance strategy their presence became less important and turned therefore to down-regulated. Compared to differentially abundant root proteins after 3 days of exposure, where no pathway or metabolism was clearly affected, protein metabolism and stress metabolism were obviously addressed after 21 days of exposure, followed by the carbohydrate metabolism. Considering the meta-analysis data, the same metabolisms were affected (see section 4.2.6.1.1.2). However, a closer look reveals some differences; (1) in the present study, protein metabolism and stress metabolism are mainly up-regulated whereas a more or less even distribution between up- and down-regulation was described in the meta-analysis, (2) carbohydrate metabolism was clearly down-regulated when considering the meta-analysis data whereas a more or less even distribution between up- and down-regulation was present in this study. Like in leaves, some unexpected down-regulations occurred. Glutamine synthetase is down-regulated in roots after 21 days of exposure, indicating that a possible shift in stress avoidance strategy would also manifest on roots. The down-regulation of major latex protein, involved in stress defence, might be explained in the same way. Upon long term Cd exposure, major latex protein was described to be down-regulated in spinach leaves as well (Fagioni and Zolla, 2009a).

To conclude, a similar study was conducted by Kieffer *et al.* (2008, 2009a, 2009b) on leaves and roots of hydroponically grown *P. tremula* L. cuttings. As in our experiment, cuttings of the Cd group were exposed to 20 µM of $CdSO_4$. Due to the height they reached after 21 days, it was not possible to prolong the growth of the cuttings used in our experiment. On the other hand, an exposure

period of 14 days did not induce visible signs of toxicity and was therefore not chosen to work with. Since Kieffer *et al.* (2008, 2009a, 2009b) chose exposure periods of 3, 7, 14, 28 and 56 days, only a comparison at 3 days of exposure was possible. In leaves, differentially abundant proteins (DAPs) were assigned in *P. tremula* L. (Kieffer *et al.*, 2008 (4 DAPs); 2009b (2 DAPs)), while none were differentially abundant in *P. deltoides x (trichocarpa x deltoides)*. In roots, 7 proteins differed in abundance in *P. deltoides x (trichocarpa x deltoides)*, while only 3 DAPs were described in *P. tremula* L. (Kieffer *et al.*, 2009b). All 3 DAPs in *P. tremula* L. were involved in stress metabolism; two were up-regulated and one was down-regulated. Since none of the proteins match those found in our experiment, it confirms the observation that plants' response to Cd is species dependent (Smeets *et al.*, 2005). However it should be taken into account that this could also be an effect of the chosen experimental setup (e.g. growth conditions of the plants like light, temperature, air humidity, ….) and proteomic workflow inherent to the different labs and that, subsequently, all these parameters contribute to the resulting Cd response that is observed (see chapter 4.2).

Comparing the results obtained in our study after exposing *P. deltoides x (trichocarpa x deltoides)* to Cd for 21 days to those reported for *P. tremula* L. (Kieffer *et al.*, 2009b) after 14 and 28 days of exposure revealed that the strong down-regulation of the energy metabolism that we described in leaves (Table 4.3.3), was not detected in *P. tremula* L.. In addition, a strong effect on the carbohydrate metabolism was described in *P. tremula* L. leaves after both 14 and 28 days of exposure, while in *P. deltoides x (trichocarpa x deltoides)* leaves only down-regulations of triosephosphate isomerase and ribulose-1,5-bisphosphate carboxylase oxygenase large subunit were detected. Furthermore, no leaf proteins involved in stress metabolism were similarly affected by Cd in both poplar species. In *P. tremula* L. roots, the glycolysis and TCA cycle were down-regulated upon both 14 and 28 days of exposure while in *P. deltoides x (trichocarpa x deltoides)* only a moderate effect on the glycolysis was present and the TCA cycle was not affected. Of the stress defence related proteins, down-regulation of major latex proteins was present in both *P. deltoides x (trichocarpa x deltoides)* (21 days) and *P. tremula* L. (14 and 28 days) and the up-regulation of glutathione peroxidase was present in *P. deltoides x*

(trichocarpa x deltoides) after 21 days and in *P.* tremula L. after 28 days of exposure. The observed differences between our study and the one conducted by Kieffer *et al.* cannot be attributed to a difference in total Cd content in leaves and roots, since they were similar in *P. deltoides x (trichocarpa x deltoides)* and *P. tremula* L (Kieffer *et al.*, 2008; 2009a).

Chapter 4.4

Effects of short and long term Cd exposure on soil grown poplar (P. deltoides x (trichocarpa x deltoides)) roots and leaves at proteome level.

4.4.1 Introduction

In order to investigate the effects of one specific variable, ideally all other variables should remain constant. Working with biological material, biological variation is however inevitable and therefore, cultivation methods providing highly controllable and reproducible conditions and rendering as low variation as possible are preferable in these cases. Using hydroponic cultivation systems, not only allows you to achieve the above mentioned desired traits (Zacchini *et al.*, 2009), but also allows to minimize working space or maximize the number of repetitions. Due to high variation in the field, molecular research is mostly performed in highly controlled laboratory conditions, rendering basic insights into molecular systems. Although gained insights only apply for highly controlled lab conditions, they often serve as basis to formulate hypothesis for future field experiments. Since differences are found between highly controlled laboratory conditions and environmental realistic field conditions (see section 4.2.6.2), extrapolations are highly questionable.

In order to get a better understanding of the basic molecular effects of phytoextraction at Cd contaminated fields, a first study was performed to reveal the effect of Cd on the proteome of *Populus deltoides x (trichocarpa x deltoides)*. For this purpose, a hydroponic cultivation system was chosen to minimize all additional variations (chapter 4.3). However, focussing on future field experiments, extrapolation of the gained insights was questioned: using hydroponic cultivation systems involves a risk at anaerobic conditions at roots level, prevents the establishment off a rhizosphere, delivers roots that are not comparable to roots formed in soils and supplies a steady and constant availability of nutrients. Moreover, it is known that plants grown in hydroponic cultivation systems accumulate more Cd than those produced in soil systems

(Grant *et al.*, 1998). Therefore, with the prospect to field experiments, the effect of Cd on the proteome of *Populus deltoides x (trichocarpa x deltoides)* cultivated in soil systems was studied.

4.4.2 Material and methods

4.4.2.1 Plant cultivation

Poplar cuttings (*Populus deltoides x* (*trichocarpa x deltoides*)) of 30 cm were planted in 4 l pots with 4 kg sandy soil on top of a drainage system (Figure 4.4.1). As in chapter 4.3, plants were incubated in a growth chamber at day/night temperature of 22/18°C with a 12 h light period, relative humidity of 65% and photosynthetic active radiation of 170 µmol m^{-2} s^{-1}. At the moment of planting, 400 ml ½ strength Hoagland's solution was added. During the next weeks, cuttings were watered three times a week with tap water and once every two weeks 200 ml ½ strength Hoagland's solution was added. When sufficient fully expanded leaves were present on all cuttings, cuttings were divided into four batches:

- Batch 1: control group, harvested 3 days after Cd exposure (control group of batch 2)
- Batch 2: Cd group, harvested 3 days after Cd exposure
- Batch 3: control group, harvested 21 days after Cd exposure (control group of batch 4)
- Batch 4: Cd group, harvested 21 days after Cd exposure

Cd groups were exposed to $CdSO_4$ at a final concentration of 40 mg kg^{-1} soil. After the start of exposure, the same watering schedule was continued. At day 3 and day 21, the according batches were harvested. Of each batch, growth parameters were determined, 5 leaf and root samples were taken for protein analysis and 5 leaf and root samples were taken to determine Cd content in plant tissue. Growth parameters were statistically analyzed using a two-way ANOVA (condition 1: time, condition 2: exposure; $p<0.05$) and post hoc multiple comparison testing (Tukey Kramer). When necessary, log-transformations were applied to approximate normality and/or homoscedasticity. Statistical analyses were performed using SAS 9.2. For soil metal content determination, 3 soil samples of each pot were taken to determine total and plant available Cd content (see section 4.4.2.2.1 and 4.4.2.2.2).

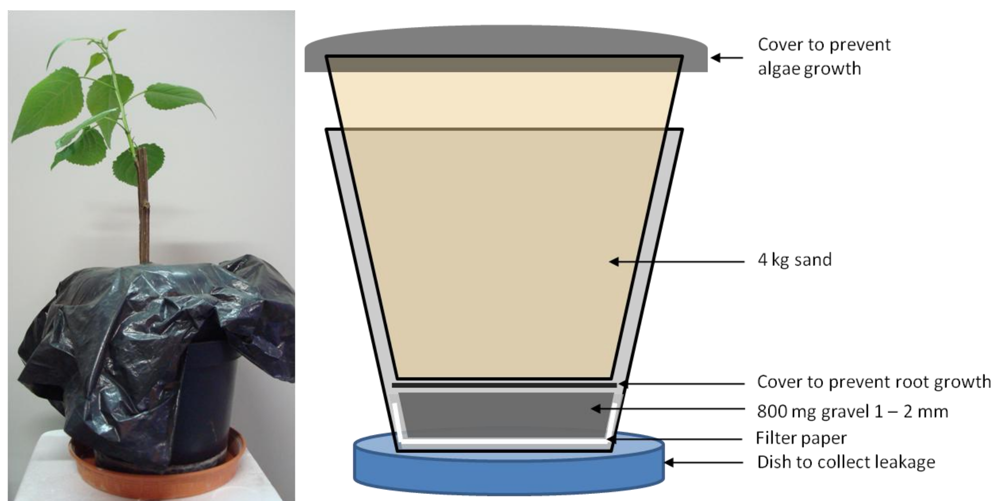


Figure 4.4.1: Soil cultivation system. Drainage system was composed of filter paper, 800 mg gravel (1 – 2 mm) and a cover to prevent roots to grow out of the soil compartment. Cuttings were planted in 4 kg sand and a cover was placed on top of the soil system to prevent algae growth. A dish was placed at the bottom to collect percolating water.

4.4.2.2 Analysis of metal content in plant tissue and soil

4.4.2.2.1 Plant available metal content in soil

From moist sandy soil, an equivalent of 5 g soil dry weight was taken for analysis. To this, 25 ml 0.1 M $Ca(NO_3)_2$ was added and samples were shaken (50 rpm) for 2 h. To filtered solutions (Whatmann 40 filter), 1 ml HNO_3 suprapur was added and samples were analysed by ICP – OES to determine Cd, Zn and Fe content.

4.4.2.2.2 Total metal content in soil

Total soil metal concentration were determined by digesting 500 mg air-dried soil in 4 ml *aqua regia* (HNO_3 supra purr / HCL supra purr : 1/3) using a microwave (Milestone, 1200 MEGA). Samples were filtered (Whatmann 40 filter) and analysed by AAS to determine Cd, Zn and Fe content.

4.4.2.2.3 Plant metal content

Each fresh sample (5 leaf and root samples per batch; see section 3.2.1) was dried at 60°C. Samples were crushed and 150 mg DW was taken for analysis. To each sample, 2 ml HNO_3 suprapur was added and samples were stored overnight at room temperature. Next, samples were heated to 110°C in order to

evaporate the remaining HNO_3 suprapur. 1 ml HNO_3 suprapur was added and evaporated twice more. Thereafter, samples were resolved in 1 ml HCl suprapur and heated in a final step to evaporate the HCl suprapur. Finally, samples were resolved in 20% HCl and measured using ICP/MS (Inductively Coupled Plasma/Mass Spectrometry) to determine Cd, Zn and Fe content. When concentrations were below the detection limit, samples were analysed on ICP - OES.

4.4.2.2.4 Statistical analysis

Metal contents were statistically analyzed using a two-way ANOVA (condition 1: time, condition 2: exposure; $p<0.05$) and post hoc multiple comparison testing (Tukey Kramer). When necessary, log-transformations were applied to approximate normality and/or homoscedasticity. Statistical analyses were performed using SAS 9.2.

4.4.2.3 Proteomic analysis

Protein extraction, protein quantification, DIGE labelling, 2-DE, analysis, digestion and identification was performed as described in chapter 3 (see section 4.3.2.3 until section 4.3.2.8). For additional information concerning the identification we refer to the supplementary data (Table S4 (leaf proteins), Table S5 (root proteins)).

4.4.3 Results

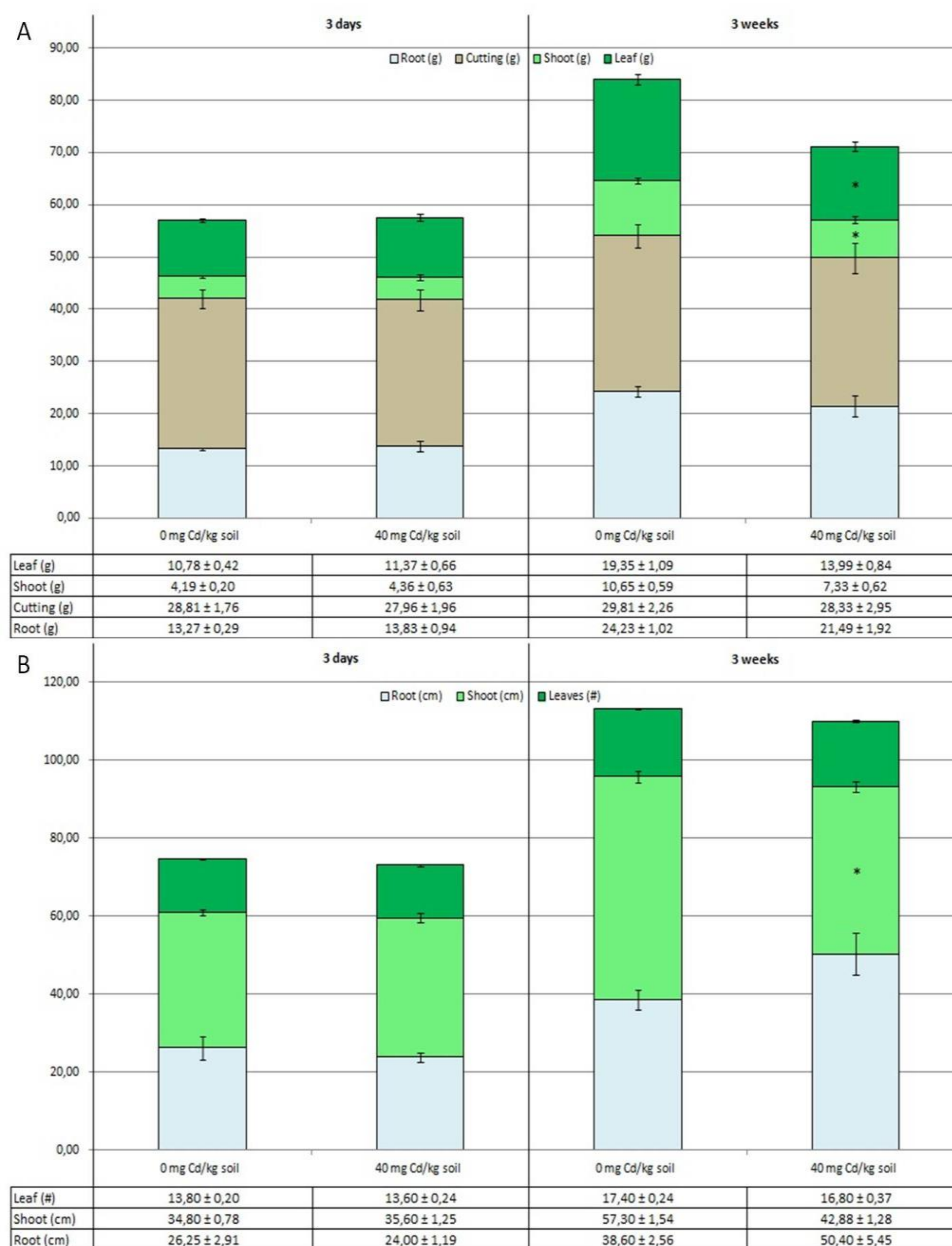
4.4.3.1 General effects

After 3 days of Cd exposure, no visible signs of stress could be observed on the poplar cuttings. However, after 21 days of Cd exposure, Cd-exposed cuttings were smaller than control cuttings, which is supported by the decreasing trend in fresh weight of shoots and roots (Figure 4.4.2A). Based on the fresh weight of leaves, a statistical difference is present between control and exposed cuttings after 21 days of exposure. On top, light chlorosis was present on the young leaves after 21 days of exposure, however no necrotic spots were present. Using leaf number, shoot length and root length as growth parameters (Figure 4.4.2B), no differences were present between control and Cd-exposed cuttings after 3 days of exposure. After 21 days of exposure Cd-exposed cuttings have significantly shorter shoots than control cuttings. Although not significant, the

increasing trend in root length after 21 days of Cd exposure is interesting. This increasing trend might be expected since roots tend to grow to less contaminated soil parts to avoid stress (Remans *et al.*, 2012).

In order to get insights in the metal accumulation within the poplar tissues, Cd, Zn and Fe were determined in leaves and roots (Figure 4.4.3). After 3 days, no significant differences were present in Cd and Zn content between control and Cd-exposed leaves (Figure 4.4.3A). After 21 days, a decreasing trend was present for Zn content in leaves when comparing control to Cd-exposed samples. This decreasing trend might indicate a competition for translocation between of Cd and Zn. In roots, no significant differences were present in Cd and Zn content between control and exposed cuttings after 3 days of exposure (Figure 4.4.3B). However, after 21 days of exposure, a significant increase in Cd content was present compared to control samples. As for leaves, a slight decreasing trend in Zn content could be observed when comparing control to Cd-exposed roots. This might be explained by the fact that the presence of Cd in the soil matrix induced competition between Zn and Cd uptake since uptake of Cd^{2+} occurs via Zn^{2+}, Fe^{2+} and Ca^{2+} transporters (Clemens S, 2006b). Due to technical errors, Fe concentration could not be estimated in leaf and root samples.

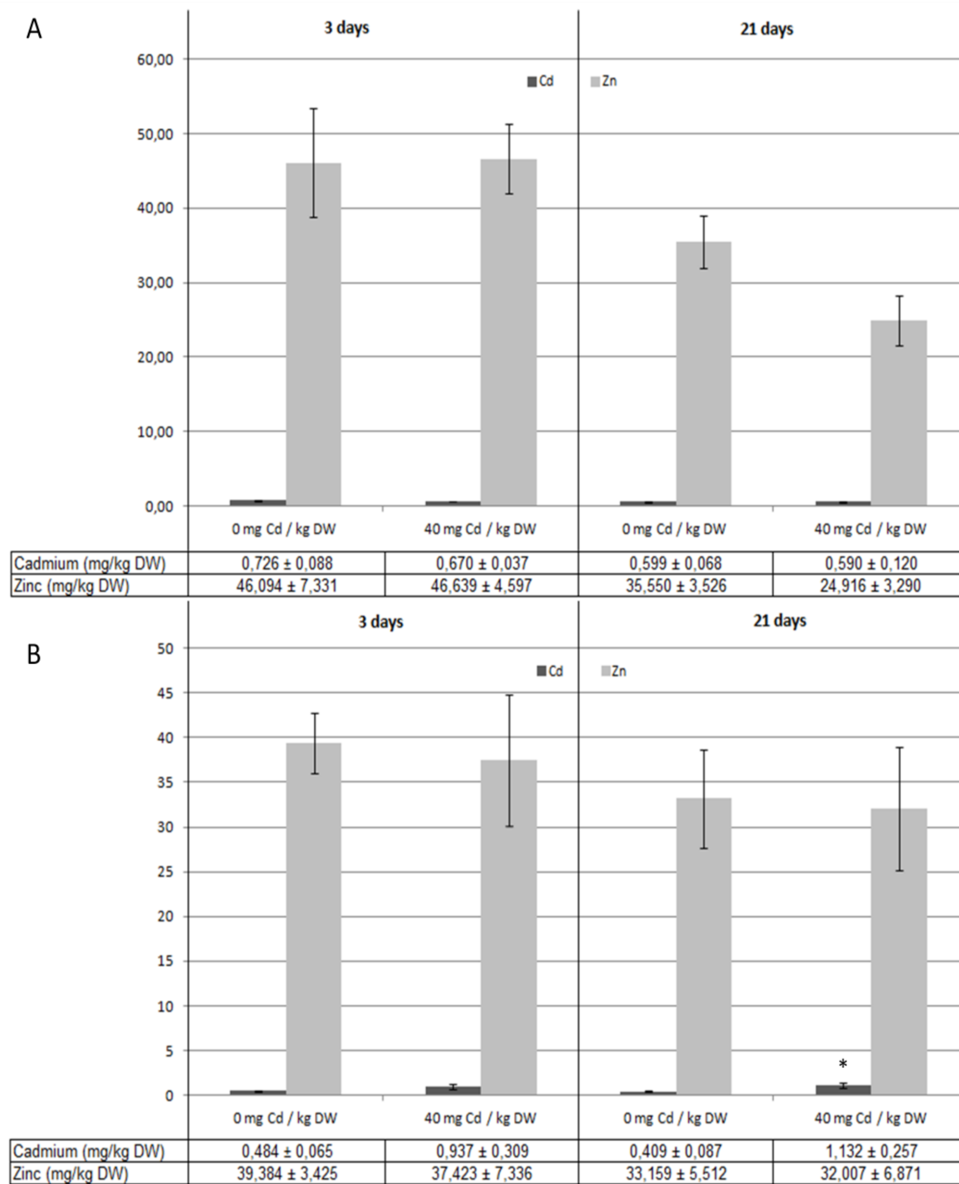
To determine total and estimate plant available metals in the soils, 3 samples were taken in each pot using a soil-sampler with 3 cm diameter. Although samples were taken over the total depth of the pot and at 3 different points, metal analysis did not provide good results. For 17 out 20 pots, the concentrations were below the detection limit. Since the values found in 3 Cd-exposed soils, were within the range of expectation, performance of the extraction and AAS technique were not questioned. Therefore, these results – or the lack of it –indicate heterogeneity within the soils.



	3 days: 0 mg Cd/kg soil	3 days: 40 mg Cd/kg soil	3 weeks: 0 mg Cd/kg soil	3 weeks: 40 mg Cd/kg soil
Leaf (g)	10,78 ± 0,42	11,37 ± 0,66	19,35 ± 1,09	13,99 ± 0,84
Shoot (g)	4,19 ± 0,20	4,36 ± 0,63	10,65 ± 0,59	7,33 ± 0,62
Cutting (g)	28,81 ± 1,76	27,96 ± 1,96	29,81 ± 2,26	28,33 ± 2,95
Root (g)	13,27 ± 0,29	13,83 ± 0,94	24,23 ± 1,02	21,49 ± 1,92

	3 days: 0 mg Cd/kg soil	3 days: 40 mg Cd/kg soil	3 weeks: 0 mg Cd/kg soil	3 weeks: 40 mg Cd/kg soil
Leaf (#)	13,80 ± 0,20	13,60 ± 0,24	17,40 ± 0,24	16,80 ± 0,37
Shoot (cm)	34,80 ± 0,78	35,60 ± 1,25	57,30 ± 1,54	42,88 ± 1,28
Root (cm)	26,25 ± 2,91	24,00 ± 1,19	38,60 ± 2,56	50,40 ± 5,45

Figure 4.4.2: Effects of Cd exposure on (A) weight of leaves, shoots and roots and on (B) shoot length, root length and number of leaves. Data presented as average ± standard error. Asterisks present significant differences (2 way ANOVA, $p<0.05$) between control and exposed cuttings of the same exposure period.



A

	3 days: 0 mg Cd / kg DW	3 days: 40 mg Cd / kg DW	21 days: 0 mg Cd / kg DW	21 days: 40 mg Cd / kg DW
Cadmium (mg/kg DW)	0,726 ± 0,088	0,670 ± 0,037	0,599 ± 0,068	0,590 ± 0,120
Zinc (mg/kg DW)	46,094 ± 7,331	46,639 ± 4,597	35,550 ± 3,526	24,916 ± 3,290

B

	3 days: 0 mg Cd / kg DW	3 days: 40 mg Cd / kg DW	21 days: 0 mg Cd / kg DW	21 days: 40 mg Cd / kg DW
Cadmium (mg/kg DW)	0,484 ± 0,065	0,937 ± 0,309	0,409 ± 0,087	1,132 ± 0,257
Zinc (mg/kg DW)	39,384 ± 3,425	37,423 ± 7,336	33,159 ± 5,512	32,007 ± 6,871

Figure 4.4.3: Metal concentrations in (A) poplar leaves and (B) roots. Data presented as average ± standard error. Asterisks present significant differences (2 way ANOVA, $p<0.05$) between control and samples of exposed cuttings of the same exposure period.

4.4.3.2 Effects of short and long term exposure to Cd on poplar

All identified proteins with a fold change > |1.5| (ANOVA < 0.05) were manually classified into functional groups. In order to facilitate comparison with the experiment on hydroponic cultivation systems (chapter 4.3), the same functional classification was used. The energy metabolism comprises light reactions of photosynthesis and mitochondrial electron transport chain. The carbohydrate metabolism includes proteins involved in (1) "CO_2-fixation", comprising carbon reactions of photosynthesis, (2) "Catabolism", comprising glycolysis/gluconeogenesis, TCA cycle, pyruvate metabolism and pentose phosphate pathway and (3) "Metabolism of C-based molecules", comprising all other carbohydrate metabolism subgroups, as listed in the KEGG PATHWAY database (http://www.genome.jp/kegg/pathway.html). Proteins to which no clear function could be assigned, were categorized into 'others'.

4.4.3.2.1 Effects on poplar leaves at proteome level

On each gel approximately 1600 spots were detected of which 1025 could be matched on 90% of all the gels. Matched spots were included in the statistical analysis identifying 779 spots to be differentially abundant (two-way ANOVA; condition 1: time, condition 2: exposure; $p<0.05$). A PCA analysis revealed a separation between samples after 3 days of exposure and samples after 21 days of exposure (Figure 4.4.4). However, no separation between Cd-exposed and control samples is present within the same exposure period. Exactly the same separation is revealed by hierarchical classification of the 779 differentially abundant proteins (Figure 4.4.5).

Of the 779 differentially abundant proteins (DAPs), only seven spots differed in abundance between control and Cd-exposed plants and showed an absolute variation of at least 1.5-fold. These proteins were picked and submitted for identification and will be discussed hereafter. In the subsequent discussion, proteins will be referred to by their protein name, as inferred by searching the accepted protein identifications for homology against NCBI non-redundant database.

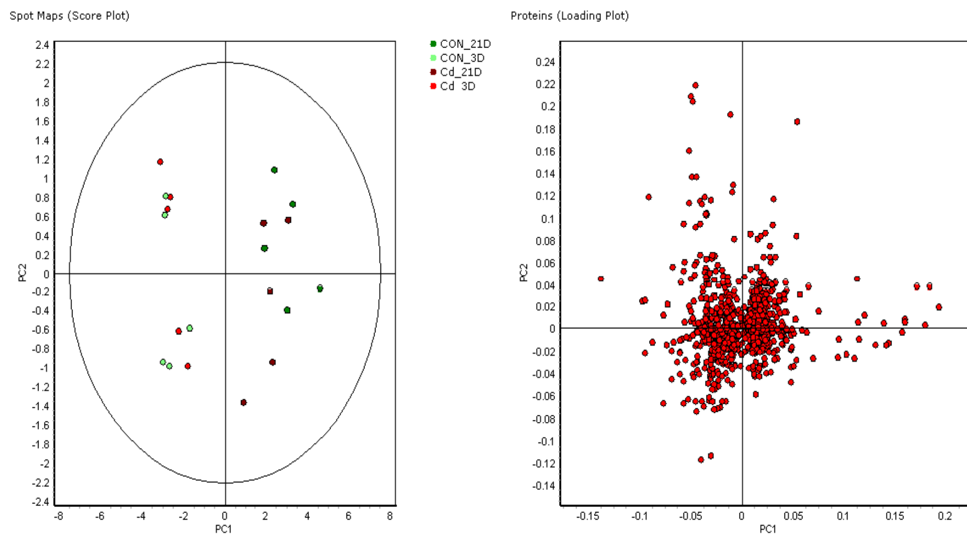


Figure 4.4.4: PCA analysis of 779 differentially abundant leaf proteins ($p<0.05$; two-way ANOVA). The left panel shows the distribution of spot maps regarding the two principal components (PC1 and PC2 explaining a cumulated 68.3% of all variation). The right panel shows the distribution of the proteins.

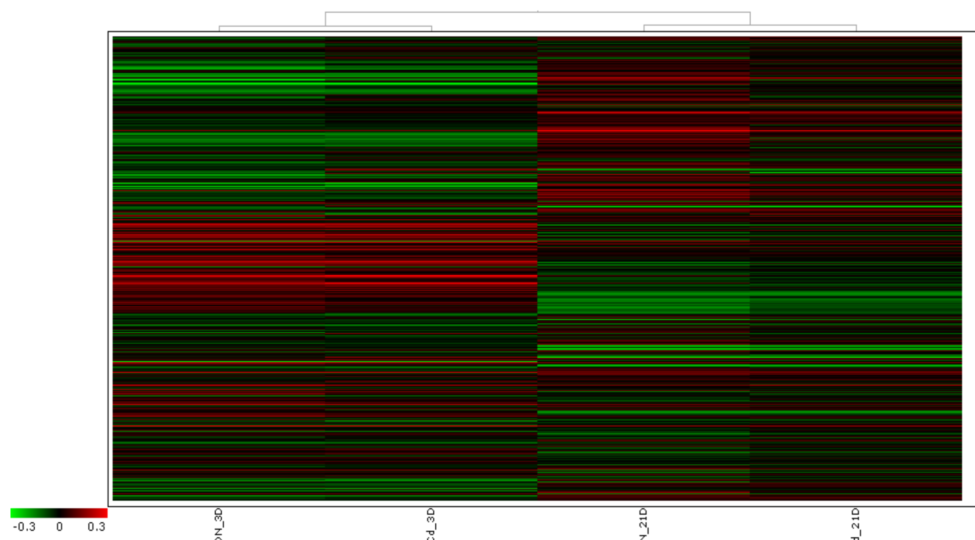


Figure 4.4.5: Hierarchical clustering of 779 differentially abundant leaf proteins ($p<0.05$, two-way ANOVA). Control and Cd-exposed groups are grouped together according to the exposure period. A clear separation of the exposure periods is present.

Of the four DAPs between control samples to samples exposed for 3 days to Cd, only two proteins could be identified unambiguously (Table 4.4.1). Elongation factor G (EF-G) is known to be a key protein in translation elongation (Nagano *et al.*, 2012). Although translocation can happen spontaneously, EF-G greatly accelerates this process (Chen *et al.*, 2012). For details on the influence of EF-G on translocation, we refer to Chen *et al.* (2012) for a review. In *E. coli* EF-G is very susceptible to oxidation and moreover, the translational mechanism is partly regulated by the redox state of EF-G which might depend on the balance between the supply of reducing power and the degree of oxidative stress (Nagano *et al.*, 2012). The up-regulation of EF-G upon Cd exposure might indicate that not only the redox state of EF-G is influenced by the degree of oxidative stress but the protein abundance as well (Table 4.4.1). Although CO_2 fixation is generally down-regulated upon Cd exposure (see section 4.2.7), RuBisCO is often reported to be up-regulated upon Cd exposure (Kieffer *et al.*, 2008; 2009a; 2009b; Fagioni and Zolla, 2009a; Durand *et al.*, 2010; Semane *et al.*, 2010; Visioli *et al.*, 2010). The up-regulation upon short term exposure, present in this study, can be seen as an attempt to maintain cellular function.

After 21 days of exposure to Cd, only three DAPs were present of which one was up-regulated and two were down-regulated. Of these, only one could be identified unambiguously and is presented in Table 4.4.1. Functional grouping revealed that only protein metabolism and carbohydrate metabolism were affected (Table 4.4.2). Heat shock proteins are known to enable cells to maintain proper folding of proteins, in both unstressed and stressed conditions (Sarkar *et al.*, 2012). Hsp70 members are involved in post-translational translocation of proteins across membranes in mitochondria (Sarkar *et al.*, 2012). Since Cd is able to induce protein confirmations by e.g. attacking disulfide bridges, up-regulation of HSP70 is likely to occur upon Cd exposure and is frequently reported (Kieffer *et al.*, 2009b; Durand *et al.*, 2010; Zhoa *et al.*, 2011). In this study, HSP70 is down-regulated confirming the Cd-induced down-regulations of this protein reported by Kieffer *et al.* (2008; 2009b) and Durand *et al.* (2010).

Table 4.4.1: List of identified proteins extracted from soil grown leaves after 3 and 21 days of Cd exposure. CON: control, CAD: cadmium.

Master No.	GI number	Protein name	Appearance	1-ANOVA	Av. Ratio CON 3 - CAD 3	Av. Ratio CON 21 - CAD 21
Protein metabolism						
114	222841862	Elongation factor G chloroplastic-like	30 (30)	5,70E-08	**1,65**	-1,1
242	222847569 222850363	Heat shock 70 kDa mitochondrial-like	30 (30)	1,10E-06	-1,16	**-1,52**
Carbohydrate metabolism						
526	158513644	Ribulose-1,3-bisphosphate carboxylase oxygenase large subunit	30 (30)	0,00018	**2,1**	1,38

Master No represents the spot number on the master gel. GI number represents the corresponding accession number in the NCBInr database. Protein name obtained via Blast2Go software by blasting against the NCBInr database. Appearance indicates the number of gels in which the spot appeared, the total gel number is given between brackets. 1-ANOVA represents the p-value with $\alpha = 0,05$. Average ratio of the protein abundance is given for 3 and 21 days of exposure, positive values are gives as such, while negative values are given according to the following formula: given value = -1/average ratio.

Table 4.4.2: Functional grouping of identified proteins extracted from soil grown leaves after 3 and 21 days of Cd exposure. Absolute numbers (#) and percentages (%) are given. Percentages are calculated per conditional group.

	3 days				21 days			
	UP		DOWN		UP		DOWN	
	#	%	#	%	#	%	#	%
Protein metabolism	1	50.0	-		-		1	100.0
Carbohydrate metabolism	1	50.0	-		-		-	
Total	**2**				**1**			

4.4.3.2.2 Effects on poplar roots at proteome level

From roots, approximately 2500 proteins were detected on each gel. Of these, 1452 proteins could be matched on 90% of all the gels and were included in the statistical variation. A two-way ANOVA (condition 1: time; condition 2: exposure) was performed on the dataset identifying only 121 spots to be differentially abundant ($p<0.05$). An absolute variation of at least 1.5-fold between proteins of control and exposed roots was detected for 42 spots and were subjected to identification. PCA analysis grouped proteins of control and roots of 3 days Cd-exposed plants together (Figure 4.4.6). Proteins of roots harvested after 21 days of exposure (control and exposed) are separated from the proteins of 3 days exposed plants along the first axis. Root proteins of control and 21 days exposed plants are separated along the second axis.

A hierarchical clustering of the 121 differentially abundant proteins reveals that after 21 days of exposure the control samples grouped closer together with the 21 days Cd-exposed ones than with the ones exposed for 3 days to Cd. (Figure 4.4.7).

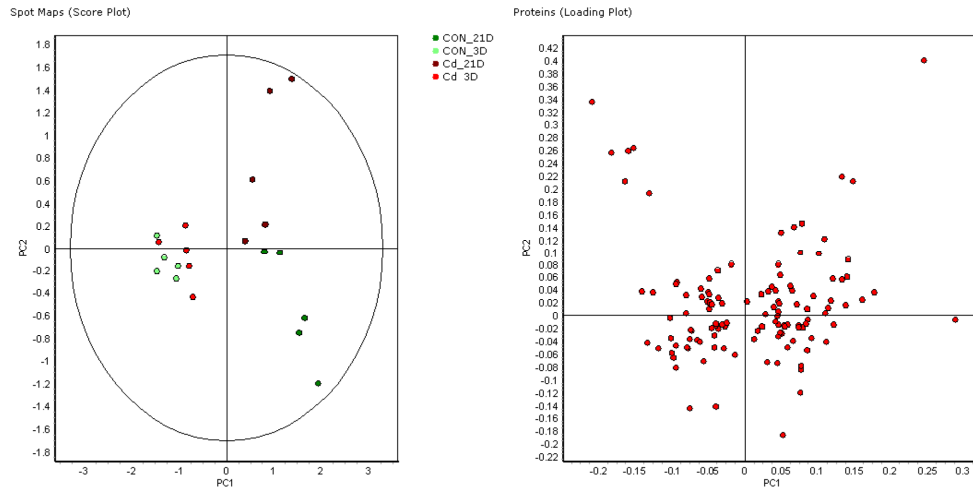


Figure 4.4.6: PCA analysis of 121 differentially abundant root proteins ($p<0.05$; two-way ANOVA). The left panel shows the distribution of spot maps regarding the two principal components (PC1 and PC2 explaining a cumulated 74.2% of all variation). The right panel shows the distribution of the proteins.

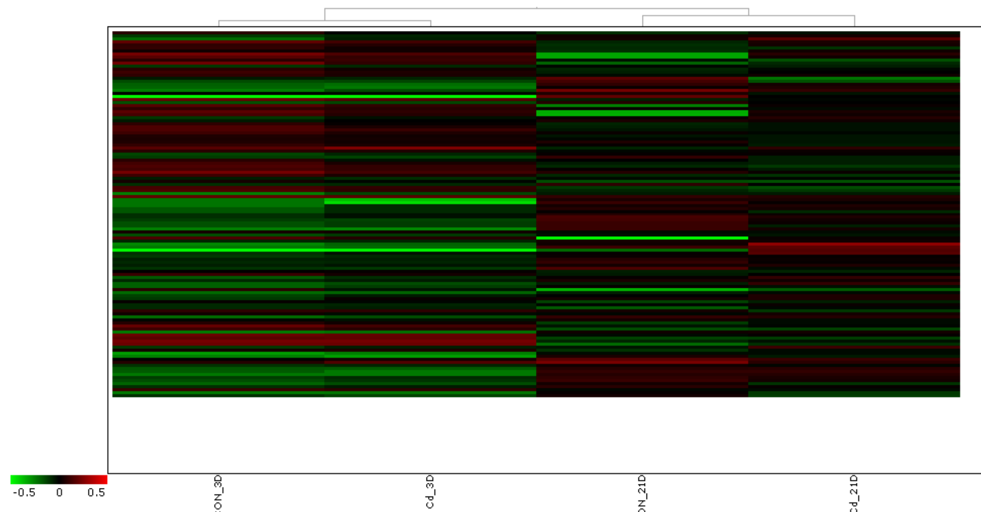


Figure 4.4.7: Hierarchical clustering of 121 differentially abundant root proteins ($p<0.05$, two-way ANOVA). Control and Cd-exposed groups are grouped together according to the exposure period. A clear separation of the exposure periods is present.

In order to map the Cd effect, a comparison between control and Cd-exposed samples was made upon 3 and 21 days of exposure. Of the 42 DAPs with an absolute abundance variation of at least 1.5-fold, only 15 could be identified unambiguously. After 3 days of exposure, only three root proteins differed in abundance, suggesting only a minor stress response (Table 4.4.3). Of these, two proteins were down-regulated and one up-regulated. Up-regulation of glycerol kinase after 3 days of Cd exposure indicates a need for energy and reducing power. That is, glycerol kinase (EC 2.7.1.30) catalyses the conversion of glycerol into sn-glycerol-3-phosphate using ATP. Next, sn-glycerol-3-phosphate can be dehydrated into dihydroxyacetone phosphate by the action of glycerol-3-phosphate dehydrogenase, which can further be metabolised into glyceraldehyde-3-phosphate (Yeh *et al.*, 2008). Glyceraldehyde-3-phosphate can subsequently be used in the glycolysis to produce energy and reducing power which is required to counterbalance Cd-induced ROS (Kieffer *et al.*, 2009a). In this light, the down-regulation of the predicted protein, which resembles the SGNH (Serine-Glycine-Asparagine-Histidine) plant lipase, is somewhat unexpected since lipases convert lipids into glycerol and fatty acids. The third differentially abundant protein in poplar roots after 3 days of Cd exposure appeared to be an oxygen-evolving enhancer protein (*OEE1*) that is down-regulated in roots of Cd-exposed plants. Although these are known chloroplastic proteins, their presence has been detected in poplar stems as well (Wassim *et al.*, 2013). Wassim *et al.* (2013) described a down-regulation of *OEE1* transcript levels in lower internodes of the stem caused by inclination in poplar plants. In addition, OEE1 is postulated to be involved in ROS detoxification via thioredoxin-like activity (Heide *et al.*, 2004). As plastids are indicated to act as sensing organelles to environmental changes and are involved in retrograde signalling (Fernandez and strand, 2008), the induced redox imbalance as a consequence of the decrease in *OEE1* transcript levels could be a consequence of such signalling (Wassim *et al.*, 2013).

After 21 days of exposure, 12 root proteins differed in abundance of which seven proteins were up-regulated and five down-regulated (Table 4.4.3). Differentially abundant proteins could be grouped into six functional classes (Table 4.4.4):

Protein metabolism (4), carbohydrate metabolism (2), stress metabolism (2), energy metabolism (1), nitrogen metabolism (1) and others (2).

Proteins involved in protein metabolism mainly function in protein folding. Luminal binding protein (BiP) was shown to bind to (1) newly synthesized, (2) incompletely assembled or (3) misfolded proteins (Denecke *et al.*, 1991). Since BiP is known to be induced upon conditions that cause the accumulation of misfolded proteins, the up-regulation after 21 days of Cd exposure was not surprising. Protein disulfide isomerase (PDI) is a multifunctional protein that facilitates the formation of correct disulfide crosslinks between cysteine residues (Wilkinson and Gilbert, 2004; Gilbert, 2011). Since Cd attacks disulfide bridges, up-regulation of PDI can be expected and was reported previously in poplar (Kieffer *et al.*, 2009b). Additionally, PDI is found to behave as a chaperone, inhibiting the aggregation of misfolded proteins, some of which contain no disulfide bounds (Wilkinson and Gilbert, 2004). Next to protein folding, one protein involved in protein synthesis differed in abundance as well. However, in contrast to the proteins involved in protein folding, glutamine synthetase was down-regulated. Glutamine synthetase (EC 6.3.1.2) incorporates ammonium into glutamate to form glutamine. Since glutamine is one of the constituents of glutathione and the glutathione pool is depleted upon Cd exposure (Semane *et al.*, 2010), up-regulation of glutamine synthetase upon Cd exposure can be seen as an attempt to replenish the glutathione pool, as described by Semane *et al.* (2010). In this view, down-regulation of glutamine synthetase seemed somewhat unexpected, however it has been reported previously in *Arabidopsis* leaves (Afefe *et al.*, 2012) and in hydroponically grown *Populus deltoides x* (*trichocarpa x deltoides*) roots (chapter 4.3). The down-regulation of glutamine synthetase might be a result of the observed down-regulation of ferredoxin-nitrite reductase which converts nitrite into ammonium. To our knowledge, up to date no differential expression of glutamine synthetase was reported in plant roots.

Table 4.4.3: List of identified proteins extracted from soil grown roots after 3 and 21 days of Cd exposure. CON: control, CAD: cadmium.

Master No.	GI number	Protein name	Appearance	1-ANOVA	Av. Ratio CON 3 - CAD 3	Av. Ratio CON 21 - CAD 21
Protein metabolism						
342	222846706	Luminal binding protein	30 (30)	0,002	-1,20	**2,05**
553	222842706	Protein disulfide isomerase	30 (30)	0,0014	-1,22	**2,07**
560	222842706	Protein disulfide isomerase	30 (30)	0,003	-1,14	**2,02**
1277	222831876	Glutamine synthetase	30 (30)	0,006	1,20	**-1,60**
	222847208					
	222849702					
	222849705					
Carbohydrate metabolism						
380	224127366	Transketolase	30 (30)	0,043	1,07	**-1,58**
	227467168					
445	224127366	Transketolase	27 (30)	0,00061	-1,02	**-2,67**
	227467168					
	227467170					
Energy metabolism						
275	222856683	oxygen-evolving enhancer protein 1 Chloroplast	27 (30)	0,023	**-1,75**	**2,00**
Stress metabolism						
454	222874491	Polyphenol oxidase	30 (30)	2,80E-06	-1,19	**-1,58**
1210	222848855	Predicted protein - Patatin-like phospholipase of plants	30 (30)	0,037	1,05	**1,67**
45	222867350	Villin 2	27 (30)	0,017	-1,07	**1,58**
49	222854917	Villin 2	27 (30)	0,029	-1,04	**1,50**
	222867350					

Table 4.4.3: List of identified proteins extracted from soil grown roots after 3 and 21 days of Cd exposure. CON: control, CAD: cadmium (continued).

Master No.	GI number	Protein name	Appearance	1-ANOVA	Av. Ratio CON 3 - CAD 3	Av. Ratio CON 21 - CAD 21
Nitrogen metabolism						
622	118487557	Ferredoxin--nitrite reductase	27 (30)	0,023	1,01	**-1,64**
	224123494					
Others						
837	222835966	Glycerol kinase	30 (30)	0,049	**1,71**	-1,20
1189	118486083	Predicted protein - SGNH plant lipase like	30 (30)	0,00034	**-1,74**	-1,21
	118487168					

Master No represents the spot number on the master gel. GI number represents the corresponding accession number in the NCBInr database. Protein name obtained via Blast2Go software by blasting against the NCBInr database. Appearance indicates the number of gels in which the spot appeared, the total gel number is given between brackets. 1-ANOVA represents the p-value with α = 0,05. Average ratio of the protein abundance is given for 3 and 21 days of exposure, positive values are gives as such, while negative values are given according to the following formula: given value = -1/average ratio.

Table 4.4.4: Functional grouping of identified proteins extracted from soil grown roots after 3 and 21 days of Cd exposure. Absolute numbers (#) and percentages (%) are given. Percentages are calculated per conditional group.

	3 days				21 days			
	UP		DOWN		UP		DOWN	
	#	%	#	%	#	%	#	%
Protein metabolism	-		-		3	25.0	1	8.3
Carbohydrate metabolism	-		-		-		2	16.7
Energy metabolism	-		1	33.3	1	8.3	-	
Nitrogen metabolism	-		-		-		1	8.3
Stress metabolism	-		-		3	25.0	1	8.3
Others	1	33.3	1	33.3	-		-	
Total	**3**				**12**			

Here, transketolase was reported to be down-regulated, confirming the down-regulation described in roots of *Populus tremula* L. (Kieffer *et al.*, 2009b). In contrast, transketolase was down-regulated after 21 days of Cd exposure in *Populus deltoides x* (*trichocarpa x deltoides*) whereas it was only down-regulated after 56 days of exposure in *Populus tremula* L. (Kieffer *et al.*, 2009b). Transketolase catalyses the conversion of D-xylulose-5P towards D-glyceraldehyde-3-phophate, making a link between the pentose phosphate pathway and glycolysis. As after 3 days of Cd exposure, OEE1 was differentially abundant after 21 days, however after 21 days of exposure it became up-regulated instead of down-regulated. Next to its function in the photosystem II complex (Ko and Cashmore, 1989), OEE1 is considered to be involved in detoxification of ROS (Wassim *et al.*, 2013), explaining its up-regulation after 21 days of exposure to Cd.

Considering proteins involved in stress metabolism, polyphenol oxidase (PPO) was down-regulated after 21 days of Cd exposure. Although the function of PPO still remains enigmatic, a role in resistance to pathogens and herbivores has been suggested (Mayer AM, 2006). Since a major focus of research in unravelling PPO function has been its potential role in defence mechanisms in plants, it is interesting that it becomes differentially abundant during Cd exposure. Moreover, in case PPO is involved in the generation of ROS (Mayer AM, 2006), its down-regulation during Cd exposure might be an attempt of the plant to decrease ROS production. Patatin-like phospholipases are suggested to

play a role in inducing plant defence responses (Dhondt *et al.*, 2000). Although patatin-like phospholipases are mainly linked to pathogen defence (Dhont *et al.*, 2000; Banerji and Flieger, 2004), their up-regulation during Cd exposure might indicate a role in abiotic stress defence as well. Villin 2 was also grouped under stress metabolism since it might have a role in avoiding Cd toxicity at root level. Villins are actin-bundling proteins which have been shown to play a role in organizing the cytoplasm in pollen tubes (Zhang *et al.*, 2011) and root hairs (Ketelaar *et al.*, 2002) as well as in nuclear positioning in root hairs (Ketelaar *et al.*, 2002). Working on *vln2* and *vln3* double mutants, van der Honing *et al.* (2012) described problems with coordinated cell elongation. They concluded that villin is involved in the generation of thick actin filament bundles and suggest that these bundles are important for the coordination of cell expansion in different organs (van der Honing *et al.*, 2012). Therefore, up-regulation of villins upon Cd exposure might be an indication for the directional root growth towards less Cd contaminated areas, as described by Remans *et al.* (2012).

4.4.4 Conclusion

An exposure period to Cd of 3 days was too short to induce visible signs of toxicity on *P. deltoides x (trichocarpa x deltoides)* shoots. Moreover, Cd was not able to induce growth reduction within this time frame (Figure 4.4.2). Although an increasing trend was present in Cd content in leaves and roots after 3 days of exposure, no significant differences were present compared to unexposed controls (Figure 4.4.3). The absence of a morphological Cd effect after 3 days of exposure was confirmed by a limited Cd effect on the proteome of leaves and roots since only little differentially expression was present. In leaves, only 4 proteins differed in abundance of which 2 were identified unambiguously (Table 4.4.1). In roots only 8 proteins showed a differential expression and of these 3 could be identified unambiguously (Table 4.4.3). These results suggest that an exposure time of 3 days is too short to induce a Cd effect in leaf and root proteomes of soil grown *P. deltoides x (trichocarpa x deltoides)* cuttings. Moreover, PCA analysis (Figure 4.4.4 and 4.4.6) and hierarchical clustering (Figure 4.4.5 and 4.4.7) confirm that no separation could be made between control and Cd gels after 3 days of exposure in both leaves and roots. Up to our

knowledge, no study was conducted to unravel the short term effect of Cd on leaf and root proteomes in soil grown plant.

After 21 days of Cd exposure, a decreasing trend in growth of *P. deltoides x (trichocarpa x deltoides)* was present. Compared to control cuttings, significant reductions were present on leaf and shoot mass as well as on shoot length (Figure 4.4.2). In leaves of Cd-exposed cuttings the Cd content was not different compared to the unexposed controls (Figure 4.4.3A). In roots, however, a significantly higher Cd content was present compared to control cuttings (Figure 4.4.3B). At the proteome level, the Cd effect was mild in leaves even after 21 days of exposure: 8 proteins differed in abundance compared to 4 DAPs after 3 days of exposure. The mild Cd effect on leaves after 21 days of exposure was confirmed based on PCA analysis (Figure 4.4.4) and hierarchical clustering (Figure 4.4.5) since no separation between control and Cd gels was present. However, in roots, a more pronounced effect of Cd was present after 21 days than after 3 days of exposure: 37 proteins differed in abundance after 21 days compared to 3 DAPs after 3 days. The more pronounced effect of Cd on roots was confirmed by PCA analysis (Figure 4.4.6) which indicated a separation between control and Cd gels after 21 days of exposure. However, based on hierarchical clustering (Figure 4.4.7) no separation was present between control and Cd gels after 21 days of exposure. The higher number of differentially abundant proteins in roots compared to leaves is contrasting to the findings in the meta-analysis of Cd-induced proteome effects in plants (see section 4.2.6.1.2). The general down-regulation of CO_2 fixation, chloroplast electron transport chain and ATP synthases in leaves, described in the meta-analysis data, was not confirmed in the leaves of our soil grown *P. deltoides x (trichocarpa x deltoides)* cuttings since only one DAP could be identified unambiguously. Compared to differentially abundant root proteins after 3 days of exposure, where no pathway or metabolism was clearly affected, protein metabolism and stress metabolism were obviously influenced after 21 days of exposure. In the meta-analysis, the same metabolisms showed to be affected (see section 4.2.6.1.2.1). However, a closer look revealed some differences; (1) stress metabolism was predominantly affected in the meta-analysis while in the present study the protein metabolism and stress metabolism are equally affected, (2) stress metabolism is exclusively up-regulated in the meta-analysis

while some down-regulation is present in the present study, (3) protein metabolism only ranks third place in the meta-analysis and is exclusively down-regulated while it is mainly up-regulated in the present study and finally (4) the carbohydrate metabolism appeared principally up-regulated in the meta-analysis while it is exclusively down-regulated in the present study.

In conclusion, to our knowledge only 1 study was conducted on the leaf proteome of soil grown poplar plants (Durand *et al.*, 2010) and 1 on roots proteomes of soil grown pea plants (Repetto *et al.*, 2003). Durand *et al.* (2010) reported the effect of Cd on the leaf proteome of soil grown *P. tremula x P. alba* (717-184 genotype) and revealed 120 DAPs after 61 days of exposure. Several differences appear between the present study and the study conducted by Durand *et al.* (2010): respectively (1) exposure times of 21 days and 61 days were chosen, (2) Cd concentrations of 40 mg kg^{-1} and 360 mg kg^{-1} were used and (3) a sandy soil and a combination of sandy soil and peat moss were used as matrix. Additionally, the used chemical form of Cd was not specified by Durand *et al.* (2010). Due to these differences it is difficult to make a comparison between the present study and the results of Durand *et al.* (2010). Repetto *et al.* (2003) studied the effect of Cd on the root proteome of *P sativum* L. after 35 days of exposure. No similarities were present between the proteins reported in this study and those reported by Repetto *et al.* (2003). This might be the result of the differences in experimental setup between both studies: respectively (1) exposure times of 21 days and 35 days were chosen, (2) Cd concentrations of 40 mg kg^{-1} and 100 mg kg^{-1} were applied and (3) $CdSO_4$ and $CdCl_2$ were used. Both studies used a sandy soil matrix, however Repetto *et al.* (2003) prewashed and sterilised the sandy soil prior to application.

Chapter 4.5

Effects of the cultivation system on the proteome of poplar (P. deltoides x (trichocarpa x deltoides)) leaves and roots.

4.5.1. Introduction

As mentioned before, the possibility to extrapolate data obtained in controlled conditions towards uncontrollable field conditions is questioned. In order to obtain information about the effect(s) of the cultivation system on the plant responses, we studied the effect of Cd on leaf and root proteome of both hydroponically (chapter 4.3) and soil grown (chapter 4.4) *Populus deltoides x (trichocarpa x deltoides)* cuttings. To make a comparison possible, important and controllable parameters were kept identical in both experiments: (1) light intensity, (2) light period, (3) temperature, (4) humidity, (5) applied nutrient solution and (6) Cd-induced growth reduction. To obtain similar Cd-induced growth reductions, the effect of different Cd concentrations on decreases of leaf, shoot and root mass was studied in soil grown poplar cuttings (results not shown). Growth reduction was calculated relative to control plants as follows:

$$\frac{growth\ under\ control\ conditions - growth\ under\ Cd\ exposed\ conditions}{growth\ under\ control\ conditions} \times 100$$

Based on these preliminary experiments a concentration of 40 mg kg^{-1} was chosen for soil grown cuttings. As presented in Table 4.5.1 the applied Cd concentration induced similar growth reductions in both cultivation systems, however a slightly higher reduction in fresh leaf weight was present for soil grown cuttings after 21 days of exposure.

Table 4.5.1: Cd-induced growth reduction of hydroponically and soil grown poplar cuttings after 3 and 21 days of Cd exposure. Growth reduction was calculated as indicated above.

Hydroponic cultivation system					
	3 DAYS		21 DAYS		
	0 µM	20 µM	0 µM	20 µM	% growth reduction
Leaf (g)	25.2	23.51	53.61	35.9	56.39
Shoot (g)	9.46	8.54	22.6	14.77	52.59
Root (g)	13.75	12.94	32.71	25.99	31.17
Soil cultivation system					
	3 DAYS		21 DAYS		
	0 µM	40 mg kg^{-1}	0 µM	40 mg kg^{-1}	% growth reduction
Leaf (g)	10.78	11.37	19.35	13.99	69.43
Shoot (g)	4.19	4.36	10.65	7.33	54.02
Root (g)	13.27	13.83	24.23	21.49	30.11

4.5.2. Effect of cultivation system on Cd content in leaves and roots

Despite the similar effects on growth parameters, great differences in Cd content were present between hydroponic and soil grown cuttings in both, leaves and roots (Table 4.5.2). Firstly, the Cd concentration is much higher in hydroponically grown leaves and roots compared to soil grown leaves and roots. Further, after 3 days of exposure Cd content in roots of hydroponically grown cuttings was 42 times higher than in control roots while in roots of soil grown cuttings the Cd content only doubled when exposed to Cd. In leaves, Cd content showed an increasing trend in hydroponically grown cuttings while a minor decrease was present in soil grown leaves after 3 days of exposure. After 21 days of exposure, Cd content in roots of hydroponically grown cuttings was 254 times higher compared to control roots while it was only increased by 3-fold in roots of soil grown cuttings. On hydroponic cultivation systems, a significant increase in Cd content was present in leaves while no effect was present in soil grown cuttings.

Table 4.5.2: Cd content (in mg kg^{-1} dry weight) in leaves and roots of hydroponically and soil grown poplar cuttings after 3 and 21 days of Cd exposure.

	Hydroponic cultivation system			
	3 DAYS		21 DAYS	
	0 µM	20 µM	0 µM	20 µM
Leaves	2.6 ± 1.0	6.7 ± 1.4	0.0 ± 0.0	154.4 ± 13.2
Roots	7.8 ± 3.7	330.4 ± 43.5	5.0 ± 3.3	1269.8 ± 164.9
	Soil cultivation system			
	3 DAYS		21 DAYS	
	0 µM	40 mg kg^{-1}	0 µM	40 mg kg^{-1}
Leaves	0.726 ± 0.088	0.670 ± 0.037	0.599 ± 0.068	0.590 ± 0.120
Roots	0.484 ± 0.065	0.937 ± 0.309	0.409 ± 0.087	1.132 ± 0.257

4.5.3. Effect of cultivation system on poplar proteome after Cd exposure

Comparing hierarchical clustering of both cultivation systems indicated that control and Cd-exposed groups clustered together after 3 days of exposure, independent of the used cultivation system (Figure 4.3.5, 4.3.7, 4.4.5 and 4.4.7). After 21 days of exposure to Cd, a separation between control and Cd-exposed groups was present for hydroponically grown leaves (Figure 4.3.5) and roots (Figure 4.3.7) proteomes. However, no separation was obtained between control and Cd-exposed groups for leaf (Figure 4.4.5) and root (Figure 4.4.7) proteomes of soil grown plants.

Comparing the differential expression between both cultivation systems, it is generally observed that more proteins are differentially abundant in leaves and roots of hydroponically grown cuttings compared to leaves and roots (Table 4.5.3) of soil grown plants. Only one exception is present: after 3 days of exposure two proteins differed in abundance in leaves of soil grown cuttings while on hydroponic cultivation systems none were differentially abundant. In hydroponically grown plants, the amount of DAPs (differentially abundant proteins) is higher in leaves compared to roots after Cd exposure, confirming the findings in the meta-analysis of Cd-induced proteome effects in plants (see

Table 4.2.5). However, data obtained in soil grown poplar plants, indicate that this general observation is not true in soil cultivation systems. As for leaves, differences were present between root proteomes of hydroponically and soil grown cuttings after 3 days of exposure to Cd (Table 4.5.3). Comparing the DAPs, only one protein, SGNH plant lipase, was differentially abundant in both cultivation systems. However, SGNH plant lipase was up-regulated in hydroponically grown roots while down-regulated in soil grown roots.

After 21 days of exposure, a general down-regulation of CO_2 fixation, chloroplast electron transport chain and ATP synthases was present in leaves of hydroponically grown cuttings. In contrast, only 1 protein was differentially abundant in leaves of soil grown cuttings upon 21 days of exposure. In roots, the same metabolisms were addressed in both cultivation systems after 21 days of exposure: protein metabolism and carbohydrate metabolism were primary addressed, followed by the stress metabolism (Table 4.5.3). However, a closer look into the protein metabolism revealed that only glutamine synthetase is differentially abundant in both cultivation systems. Moreover, it was down-regulated in both cultivation systems. Since glutamine synthetase uses ammonium to produce glutamine, the down-regulation of glutamine synthetase in soil grown roots can be explained by the accompanied down-regulation of ferredoxin-nitrite reductase which converts nitrite into ammonium. In both cultivation systems, no protein involved in carbohydrate metabolism and stress metabolism was found to be similarly differentially abundant.

Table 4.5.3: Functional grouping of differentially abundant, identified leaf and root proteins in both cultivation systems after 3 and 21 days of Cd exposure. Absolute numbers (#) and percentages (%) are given. Percentages are calculated per conditional group.

	3 days				21 days			
	UP		DOWN		UP		DOWN	
	#	%	#	%	#	%	#	%
Hydroponically grown leaves								
Carbohydrate metabolism	-		-		-		5	13.2
Energy metabolism	-		-		-		22	57.9
Protein metabolism	-		-		2	5.3	1	2.6
Stress metabolism	-		-		-		4	10.5
Nucleotide metabolism	-		-		-		1	2.6
Others	-		-		2	5.3	1	2.6
Total	**0**				**38**			
Soil grown leaves								
Carbohydrate metabolism	1	100.0	-		-		-	
Protein metabolism	1	100.0	-		-		1	100.0
Total	**2**				**1**			
Hydroponically grown roots								
Carbohydrate metabolism	-		1	16.7	2	9.5	3	14.3
Protein metabolism	1	16.7	1	16.7	5	23.8	1	4.8
Stress metabolism	1	16.7	-		3	14.3	2	9.5
Cell - Structure, growth and maintenance	1	16.7	-		1	4.8	-	
Nucleotide metabolism	-		-		1	4.8	-	
Others	-		2	33.3	7	33.3	1	4.8
Total	**7**				**26**			
Soil grown roots								
Carbohydrate metabolism	-		-		-		2	22.2
Energy metabolism	-		1	50.0	1	11.1	-	
Protein metabolism	-		-		3	33.3	1	11.1
Stress metabolism	-		-		3	33.3	1	11.1
Nitrogen metabolism	-		-		-		1	11.1
Others	1	50.0	1	50.0	-		-	
Total	**3**				**12**			

4.5.4. Effect of cultivation system on poplar proteome of control samples in time.

Since one might argue that the different responses of the poplar proteome of leaves and roots to Cd exposure in both cultivation systems are the result of differences in Cd contents in these organs, an additional comparison was made between controls of both cultivation systems in function of time. DAPs between 3 and 21 days of control growth were listed in order to get an idea of the effect of time in each cultivation system (supplementary data; Table S6). Comparing the amount of DAPs, a huge difference was found between leaves of hydroponically and soil grown cuttings (Table 4.5.4): in leaves of hydroponically grown cuttings only 9 DAPs were reported while 94 were reported in case of soil culture. For roots, more or less similar amounts of DAPs were found (Table 4.5.4). These observations are in line with the PCA data: in hydroponically grown leaves, only a slight separation was present between control gels of 3 days and 21 days (Figure 4.3.4), while a clear separation was present in hydroponically grown roots (Figure 4.3.6), soil grown leaves (Figure 4.4.4) and soil grown roots (Figure 4.4.6)

In leaves of hydroponically grown cuttings, stress metabolism was mainly addressed, while in leaves of soil grown cuttings carbohydrate metabolism was mainly affected followed by energy metabolism and protein metabolism (Table 4.5.4). Next, the different functional groups were considered more in detail in order to explore the similarity or dissimilarity in DAPs. Focussing on energy metabolism, both DAPs in leaves of hydroponically grown cuttings were also found in soil grown leaves, however ATP synthase cf1 beta complex was up-regulated in leaves of hydroponically grown cuttings while it was down-regulated in case of soil cultivation. Carbohydrate metabolism and protein metabolism were highly addressed in leaves of soil grown cuttings while no effects were present on leaves of hydroponically grown cuttings. Considering stress metabolism, beta-1,3-glucanase was up-regulated in leaves independent of the cultivation system. No other similarities were found between other stress involved DAPs.

Table 4.5.4: Functional analysis of identified proteins, differentially abundant between control samples after 3 and 21 days of growth. Absolute numbers (#) and percentages (%) are given. Percentages are calculated per conditional group.

	UP		DOWN	
	#	%	#	%
Hydroponically grown leaves				
Energy metabolism	1	11.1	1	11.1
Stress metabolism	3	33.3	1	11.1
Nitrogen metabolism	-		1	11.1
Others	1	11.1	1	11.1
Total		**9**		
Soil grown leaves				
Carbohydrate metabolism	9	9.6	21	22.3
Energy metabolism	-		20	21.3
Protein metabolism	16	17.0	3	3.2
Stress metabolism	6	6.4	7	7.4
Nucleotide metabolism	1	1.1	2	2.1
Others	6	6.4	3	3.2
Total		**94**		
Hydroponically grown roots				
Carbohydrate metabolism	4	44.4	2	22.2
Protein metabolism	1	11.1	9	100.0
Stress metabolism	1	11.1	1	11.1
Cell - structure, growth and maintenance	1	11.1	1	11.1
Others	4	44.4	6	66.7
Total		**30**		
Soil grown roots				
Carbohydrate metabolism	3	7.7	-	
Energy metabolism	-		3	7.7
Protein metabolism	3	7.7	12	30.8
Stress metabolism	3	7.7	1	2.6
Others	7	17.9	7	17.9
Total		**39**		

In roots of both hydroponically and soil grown cuttings, protein metabolism was primary addressed and in both systems it appeared to be mainly down-regulated. Three proteins involved in protein metabolism differed in abundance in both cultivation systems, although not necessarily in the same way: (1) S-adenosylmethionine synthetase was up-regulated in roots of hydroponically grown cuttings while it was down-regulated in case of soil cultivation, (2) glutamine synthetase was clearly down-regulated in roots of hydroponically grown cuttings while was up-regulated in roots of soil grown cuttings; in contrast (3) Kunitz trypsin inhibitor 3 was down-regulated in roots grown in both cultivation systems. Focussing on carbohydrate metabolism revealed that only transketolase was differentially abundant in root tissue obtained from both cultivation systems. Moreover it was up-regulated in both systems. Of stress metabolism, no proteins were found to be differentially abundant in both cultivation systems. Finally, one other protein, cinnamyl alcohol dehydrogenase-like protein was found to be differentially abundant in roots obtained from both cultivation systems, however it was down-regulated in hydroponically grown roots and up-regulated in soil grown roots.

4.5.5. Conclusion

Recently, suggestions about the differences in responses by the types of cultivation systems were made (Durand *et al.*, 2010), questioning the possibility to extrapolate data in hydroponic cultivation systems towards field experiments. In order to tackle this question, we conducted a large scale experiment in which *P. deltoides x (trichocarpa x deltoides)* cuttings were exposed to Cd on hydroponic (chapter 4.3) and soil (chapter 4.4) cultivation systems. Except from the used cultivation systems, all parameters were kept identical in both experiments. Since it is impossible to match Cd concentrations in both leaves and roots between hydroponic and soil cultivation systems, the applied Cd concentrations were chosen to induce similar growth reductions in both cultivation systems. However, after determining the Cd concentrations in leaves and roots, it became clear that very different Cd concentrations in roots and leaves are coupled with similar growth responses in hydroponic and soil cultivation systems. In soil grown poplar cuttings, lower Cd concentrations in leaves and roots are coupled with similar growth reductions than these present

in hydroponically grown cuttings. This might indicate (1) that soil grown cuttings are more susceptible to Cd stress than hydroponically grown cuttings, (2) that subcellular localisation differ in both cultivation systems or (3) that a difference in Cd complexation/speciation exists between both cultivation systems.

When considering the effects of Cd on the proteomes of leaf and roots, we concluded that the effects in hydroponically grown cuttings differed from these in soil grown cuttings. After 3 days of exposure, no proteins were similarly differentially abundant in both cultivation systems. Although the same metabolisms were addressed in hydroponically and soil grown roots after 21 days of Cd exposure, only one protein was correspondingly differentially abundant in both cultivation systems. Additionally, when comparing the effects of the two cultivation systems on the DAPs in time on control samples, only four proteins, light-harvesting complex II protein lhcb1 and beta-1,3-glucanase in leaves and transketolase and Kunitz trypsin inhibitor 3 in roots, were found to be similarly affected in both cultivation systems. This experiment confirms that caution should be taken when extrapolating data obtained in hydroponic cultivation systems towards field conditions. Although hydroponic cultivation systems can be very useful for unravelling fundamental research questions, we strongly recommend to include soil cultivation systems in studies with prospects to future field application.

Finally, a closer look to the DAPs between 3 and 21 days of growth indicates that leaves of soil grown appeared to display some stress symptoms since CO_2 fixation and energy production were down-regulated. Of the stress metabolism, mainly proteins involved in stress defence were differentially abundant. These observations support the above mentioned hypothesis that soil grown cuttings are more susceptible to Cd stress compared to hydroponically grown cuttings.

Section V:

GENERAL DISCUSSION, CONCLUSIONS AND PERSPECTIVES

5.1 Introduction

It is estimated that by 2030 up to 190 000 km² agricultural land in the European Union will be devoted to biomass production for biofuel or bio-energy use (European Biofuels Technology Platform 2010, Innovation driving a sustainable biofuel industry, http://www.biofuelstp.eu/). To avoid competition for land between food and bio-energy production, marginal and/or contaminated lands should be exploited for bio-energy production (Weyens *et al.*, 2009). Contaminated lands are often unsuitable for food production due the risk for transfer and accumulation of toxic substances into the food chain. Taking (moderately) contaminated land into use for non-food biomass production may obviate the problem of land competition (Remans *et al.*, 2012). Re-using these contaminated lands for non-food crops, combined with soil (phyto)remediation might return them into beneficial and sustainable use and reduce detrimental social, environmental and economic impacts on affected communities (Mench *et al.*, 2009; Vangronsveld *et al.*, 2009). Obviously, plant growth and activity would be negatively affected due to the toxicity of the contaminants. Therefore, research focussing on improving plant growth and activity on (moderately) contaminated soils is essential for the implementation of non-food crops on (moderately) contaminated soils. In the past, several studies indicated that microorganisms can improve plant growth on contaminated substrates (Chen *et al.*, 2013; Srivastava *et al.*, 2012; Weyens *et al.*, 2010a, 2010b). Increasing plant biomass on (moderately) contaminated soils, not only provides a higher economic return, but also increases the remediation potential due to the 'dilution effect'. Increasing the remediation potential is an important topic since phytoremediation still suffers from a lack of economic interest due to the long implementation time needed. Endophytic bacteria have been proven to increase phytoremediation efficiency of organic contaminants (Barac *et al.*, 2004; Taghavi *et al.*, 2005; Andria *et al.*, 2009; Weyens *et al.*, 2009b; 2010a; 2010b; 2011b). Focussing on organic contaminants, Weyens *et al.* (2009b, 2009c, 2010a,) demonstrated that the use of endophytic, plant growth-promoting bacteria (PGPB), equipped with the pTOM plasmid, are able to reduce the TCE evapotranspiration (Weyens *et al.*, 2009b). Moreover, they demonstrated that prolonging the contact time between the engineered PGPB and the organic contaminant is important to enhance the degradation of the contaminant and

subsequently lowering evapotranspiration. Compared to organics, decontamination of metals is less straightforward since they are not degradable. To increase metal removal from contaminated soils, metals need to be taken up by the plants through the roots and translocated to harvestable plant parts. By 'concealing' their presence in plant tissues, more metals can be taken up before toxic effects will manifest. Keeping in mind the diffuse Cd contamination present in the north eastern part of Belgium, the initial main objective of this work was to concentrate on enhancing phytoextraction efficiency of Cd and poplar growth on Cd contaminated sandy soils by using PGPBs equipped with a metal sequestration system. Therefore, a first part of this work was dedicated to obtaining plant-associated bacteria that can improve plant growth and Cd uptake. Observing differences in toxicity responses of poplar to Cd in hydroponically and soil cultivation systems lead us to examine more in detail the underlying mechanisms of Cd toxicity using a proteomic approach.

5.2 General discussion

In this work, a first step towards a better understanding of the mechanisms of bacterial enhanced phytoextraction was to obtain plant bacteria that are able to induce plant growth and enhance phytoextraction of Cd. Poplar growth induction was already described for *P. putida* W619 and *Enterobacter sp.* E638 (Taghavi *et al.*, 2005; Taghavi *et al.*, 2010). Enhancing phytoextraction efficiency of Cd is aimed to be achieved by equipping the PGPBs with the CZR operon, as described in chapter 3.1. Equipping *P. putida* W619 with the CZR was more straightforward than it was for *Enterobacter* sp. E638 (Table 3.1.4). Upon triparental conjugation, not only a transfer of the CZR operon was observed; transconjugant strain E1600-11 inherited the ability to produce siderophores from the parental donor strain. Loss of traits is commonly observed as well (Table 3.1.5) and therefore transconjugant strains should always be tested again for their phenotypical characteristics. Prior to *in planta* experiments, transconjugant strains were tested for their ability to (1) extract Cd from the medium and to (2) produce metal-mobilizing metabolites that enhance Cd mobility in the soil. Although all tested transconjugant strains were able to increase Cd removal from the medium, only strain W1366-5 had a significant effect. By measuring the difference in pH of the culture medium during the

experiment, we were able to indirectly indicate that the CZR operon was active in transconjugant strain W1366-5 (Figure 3.1.4). Although the pH difference induced by transconjugant strain E1600-11 also suggests an activation of the CZR operon, this effect cannot be indisputably assigned to the activation of the CZR operon since its parental strain *Enterobacter* sp. E638 is also able to induce a comparable pH difference (Figure 3.1.4). Since metal mobilization experiments were able to predict effects on *S. caprea* more reliable than plant growth-promoting tests (Kuffner *et al.*, 2010), we subjected our transconjugant strains to the same test using soil from a contaminated site in Lommel containing 5 mg kg^{-1} Cd. In contrast to the results of Kuffner *et al.* (2010) our results clearly indicate a pH decrease when comparing initial pH with pH measured after the experiment. All tested transconjugant strains showed to immobilize Cd in the soil (Figure 3.1.5). Although not significant, strain W1366-5 immobilized less Cd compared to the other strains. Based on the results of this mobilization test, the transconjugant strains seemed not suitable for increasing phytoextraction efficiency. However, based on the phenotypical characteristics, the growth-promoting capacities of the parental acceptor strains and the results from the Cd extraction test in liquid medium, we still expect some positive results *in planta*. The greenhouse experiment indicates that equipping PGPBs with the CZR operon might have a positive effect on the plants' ability to extract Cd from contaminated sandy soils. Although transconjugant stain W1366-5 seemed the most promising based on the lab tests, transconjugant strain E1600-11 seemed most promising for increasing Cd phytoextraction from soils based on the greenhouse experiment; this indicates that the preceding laboratory tests, on which the selection of bacteria was based, cannot unambiguously predict the effect *in planta*.

Like mentioned earlier, observing the differences in toxicity responses of poplar to Cd in different cultivation systems (hydroponics and soils) lead us to examine more in detail the underlying mechanisms Cd toxicity. For this purpose, a proteomic approach was chosen to work with. After optimizing the technique (chapter 4.1), a thorough literature review was made to postulate a well-based hypothesis regarding the effect of Cd on plants. Retrieving the proteomic information appeared to be quite challenging and therefore the encountered obstacles were discussed in the first part of the meta-analysis (chapter 4.2).

Next, we proposed some guidelines to better standardize experiments and data publications in order to make future meta-analysis of proteomic data more straightforward.

To map the Cd effect on plants, the Cd response in both, roots and leaves, was compared between short and long term exposures and between hydroponic and soil cultivation systems. We like to emphasise that each study must be seen as a 'snapshot' of the total proteome and that all studies add proteins to the global picture that displays the Cd effect. Moreover, we must be aware that up to date only a limited part of the total 'Cd-induced proteome' is available and an overall conclusion on Cd responses at proteome level is therefore difficult to postulate. In our meta-analysis, the general idea that root proteomes change more drastically since they are in direct contact with the contaminant is clearly contradicted for plants grown in hydroponic cultivation systems (see section 4.2.6.1.1). However, after short term exposure, the root proteome appears to be highly affected in soil grown plants. In hydroponically grown plants, the 'delayed effect' manifests on both leaf and root proteomes while in soil systems, this effect appears to be absent (Table 4.2.5). Based on the functional classification, different metabolic pathways are induced after short term and long term exposure. In hydroponically grown plants, pathways are mostly moderately addressed upon short term Cd exposure while they become dramatically affected after long term exposure. As previously reported by Kieffer *et al.* (2009), in roots of soil grown cuttings, an equilibrium is established after an initial 'alarm phase'. For leaves of soil grown cuttings no conclusion can be drawn since no research was conducted on short term Cd effects on soil grown leaves. In addition, leaves and roots induce different pathways after short term and long Cd exposure.

Data presented in this meta-analysis were compared to the working model proposed by Villiers *et al.* (2011), indicating that this working model is only a general approach and does not hold stand when considering the different conditional groups (short/long term exposure, hydroponic/soil cultivation systems).

One of the main conclusions of the meta-analysis is that discrepancies exist between hydroponically and soil grown plants, as was previously suggested by Durand *et al.* (2010). However, since this meta-analysis takes into account all

proteomic studies performed on leaves and roots of Cd-exposed plants, the observed differences cannot exclusively be assigned to the cultivation method since huge differences between experimental setups are present. Working on phytoremediation, an *in situ* technique, we are highly interested whether or not results obtained in hydroponic cultivation systems can be extrapolated towards field conditions. Therefore we decided to first investigate the effect(s) of cultivation systems on the plants' proteome upon Cd exposure. For this purpose, an experiment was set up in which all parameters were kept identical except the cultivation system.

In the first part of the experiment, the effects of Cd on leaves and roots of hydroponically grown poplar plants were investigated (chapter 4.3). An exposure period of 3 days appeared to be too short to induce visible symptoms of toxicity or growth inhibition (see section 4.3.3.1). In leaves, only a minor increasing trend in Cd content was present (Figure 4.3.3) and subsequently, no proteins were differentially abundant in leaves of cuttings exposed to Cd for 3 days (Table 4.3.2). As mentioned by Kieffer *et al.* (2008, 2009b), this indicated the occurrence of the 'delay effect' in leaves of hydroponically grown *P. deltoides x (trichocarpa x deltoides)* cuttings. In roots, only 7 proteins were differentially abundant after 3 days of exposure and no specific pathway was up- or down-regulated (Table 4.3.4). Comparison with the differentially abundant proteins described by Kieffer *et al.* (2008, 2009b) in *P. tremula* L. leaves and roots exposed to the same Cd concentration for 3 days, confirmed that plants' response and sensitivity to Cd might be species dependent.

After 21 days of exposure, visible signs of toxicity, a decreasing trend in plant growth and significant increases in Cd content in leaves, shoots and roots were present (Figure 4.3.2). Like in the meta-analysis, more proteins were differentially abundant in leaves than in roots of hydroponically grown poplar cuttings. Functional analysis of differentially abundant leaf proteins (Table 4.3.3) confirms the down-regulation of CO_2 fixation, chloroplast electron transport chain and ATP synthases that we described in the meta-analysis. The stress metabolism was however addressed in a different way compared to the meta-analysis. The unexpected down-regulation of glutamine synthetase and high chlorophyll fluorescent 109 protein are thought to be a result of a shift in stress avoidance strategies. Although the addressed pathways by the root proteome of

21 days exposed hydroponically grown poplar cuttings (Table 4.3.5), are similar to those described in the meta-analysis, a closer look revealed some discrepancies. As in leaves, glutamine synthetase is down regulated after 21 days of exposure, indicating that a possible shift in stress avoidance strategy might also occur in roots.

In the second part of the experiment, the effects of Cd on leaves and roots of soil grown poplar cuttings were mapped (chapter 4.4). Again, an exposure period of 3 days appeared to be too short to induce growth inhibition or visible signs of toxicity (Figure 4.4.2). In leaves and roots, an increasing trend of the Cd concentration was present (Figure 4.4.3). This slight increase was confirmed by changes in the proteome: 4 DAPs were present in leaves and 8 DAPs were present in roots after 3 days of Cd exposure (see section 4.4.3.2.1 and 4.4.3.2.2). After 21 days of exposure, a decreasing trend in plant growth was present (Figure 4.4.2), accompanied with a significant increase of the Cd concentration in roots (Figure 4.4.3). At this exposure time, the Cd concentration in the leaves of exposed plants was the same as in the unexposed control (Figure 4.4.3). However, a moderate effect on the proteome level in the leaves was present (see section 4.4.3.2.1). As could be expected from the significant increase of Cd content in roots, a stronger effect on the root proteome was present (see section 4.4.3.2.2). However, based on hierarchical clustering (Figure 4.4.7) no separation could be made between control and Cd gels after 21 days of exposure. As described in the meta-analysis, protein metabolism and stress metabolism were obviously addressed (Table 4.4.4). However a closer look into the DAPs revealed some differences between the present study and the meta-analysis data. To our knowledge, only 2 other studies focussed on leaf and root proteomes of soil grown plant. However, due to the great differences in experimental setup between those studies and the present study, a comparison was not possible.

Finally, in order to address the question if extrapolation of hydroponically obtained data towards field experiments is allowed, the results obtained from the hydroponic experiment (chapter 4.3) and the soil experiment (chapter 4.4) were compared to each other (chapter 4.5). From this comparison we concluded that Cd-induced effects on leaf and root proteomes were different between both cultivation systems. In leaves, no protein was similar affected in both cultivation

systems upon Cd exposure. In roots, the same was observed comparing hydroponically and soil grown cuttings after 3 days of exposure. However, after 21 days of exposure one protein (glutamine synthetase) was similarly addressed in both cultivation systems (Table 4.3.4 and 4.4.3). When comparing the effect of the cultivation system on the DAPs in time on control cuttings, only 2 leaf proteins (light-harvesting complex II protein and beta-1,3-glucanase) were similar addressed independent of the cultivation system. In roots, also 2 proteins (transketolase and Kunitz trypsin inhibitor 3) were similar addressed independent of the cultivation system (Supplementary data Table S6). Moreover, the results indicated that soil grown cuttings were more susceptible to Cd stress than hydroponically grown cuttings. Since we feel that growing cuttings in soils is a better approximation for toxicity plants are experiencing in the field, we recommend to include soil cultivation systems in studies aiming extrapolation to field application. Ideally, research which focuses on techniques applicable in the field, should be mainly conducted on test sites in the field despite the presence of uncontrollable conditions.

5.3. Perspectives

Data obtained in chapter 3.1 demonstrate that the concept of equipping PGPBs with appropriate mechanisms to enhance phytoextraction can be applicable for Cd contaminated soils. To examine whether or not the observed growth stimulation and the increasing trend in Cd uptake might be significant, the experiment should be repeated with sufficient biological replicas. The most promising transconjugant strains should subsequently be tested in field trials in order to examine if they are able to establish in the field and enhance phytoextraction on sandy soils with mixed contamination. Since horizontal gene transfer is a naturally occurring mechanism, PGPBs engineered using this principle can be applied in the field without breaking the Belgian law on GMOs. By consequence, the PGPBs engineered in this work can be used for field experimentation.

Although Durand *et al.* (2010) already expressed their concerns about using data obtained in hydroponic cultivation studies to postulate hypothesis for field trials, to our knowledge no other research has been conducted up to date to confirm or reject their concerns. The results obtained from the meta-analysis

(chapter 4.2) and the comparison of effects of different cultivation methods on the plants' proteome (chapter 4.5) confirm the concern of Durand *et al.* (2010). Therefore, we strongly recommend to not make extrapolations from results obtained in hydroponic cultivation experiments towards field trials. As a consequence, we should start questioning if the advantages inherent to the hydroponic cultivation systems (e.g. lower costs and lower labour intensity compared to soil cultivation systems) balance out the fact that results obtained on hydroponic cultivation systems cannot be linked to and by consequence are not representative for those obtained on soils. Since there is still a substantial difference between soil cultivation systems in controlled environments and field trials, a comparison between those two cultivation methods should be conducted to find out whether or not results of both systems can be linked. For this purpose, as much as possible parameters should be kept the same: (1) in the soil cultivation system soil from the field site should be used, (2) the greenhouse should be close to the field site in order to reduce the difference in illumination, photoperiod, etc, (3) cuttings in the greenhouse experiment should be watered at the same rate as the observed rainfall, (4) watering should be performed using rain water collected close to the field site and (5) temperature, light intensity and humidity in the greenhouse experiment should be kept as close as possible to those occurring at the field site. While interpreting the results, it should be kept in mind that thigmomorphogenic stimulation of plants is very low in the greenhouse and also that UV irradiation cannot enter the greenhouse since glass is known to absorb almost all UV illumination. In our research we focussed only on soluble proteins and therefore it would be interesting to evaluate the effect of cultivation systems on membrane proteins as well.

With the prospect on improving phytoextraction efficiency of Cd contaminated soils, the mechanisms underlying the effects obtained by the engineered PGPBs (chapter 3.1) are still of major interest to our research group. Therefore, proteomic analysis of (1) control – not inoculated poplar cuttings, (2) control – inoculated poplar cuttings, (3) Cd-exposed – not inoculated poplar cuttings and (4) Cd-exposed – inoculated poplar cuttings should be performed in a future experiment. In this way, we will be able to point out which mechanisms are addressed by the engineered PGPBs and these insights will provide us information that can be further exploited to improve phytoextraction efficiency

even more. For example, supposing that the engineered PGPBs mainly enhance phytoextraction by enhancing the production of stress detoxification proteins, future research might focus on how to boost those proteins without the presence of bacteria. Moreover, obtained data should be tested in a full 'omics' approach.
Further, increasing attention is recently paid to the use of microbial consortia (Weyens *et al.*, 2009a). Using different microorganisms, endo- and/or rhizospheric/epiphytic, more beneficial results could be obtained as demonstrated by Langella F. (personal comments). With this in mind, it might be worthwhile to combine different engineered PGPBs in consortia in order to obtain an even higher phytoextraction efficiency.
Further, since Mertens *et al.* (2006) demonstrated that Cd concentration in willow (*Salix* sp.) leaves appears to change throughout the different seasons, we'd like to point out that more research should be conducted in order to determine the most optimal harvest time of plants used for phytoextraction. In this way, another aspect to enhance phytoextraction efficiency can be addressed optimally.

Section VI:

LITERATURE

∴ Achkor H, Diaz M, Fernández MR, Biosca JA, Parés and Martínez MC (2003) Enhanced formaldehyde detoxification by overexpression of glutathione-dependent formaldehyde dehydrogenase from Arabisopsis. *Plant Physiology*, **132**, 2248 – 2255.

∴ Afef NH, Houda G and Chiraz CH (2012) Response of Arabidopsis thaliana, seedlings to cadmium in relation to ammonium availability. *Bulletin of Environmental Contamination and Toxicology*, **89**, 1175 – 1180.

∴ Agency for Toxic Substances and Disease Registry (ATSDR). CERCLA Priority list of hazardous substances that will be the subject of toxicological profiles and support document. Atlanta (USA): ATSDR, U.S. Department of Health and Human Services, 2007.

∴ Aina R, Labra M, Fumagalli P, Vannini C, Marsoni M, Cucchi U, Bracale M, Sgorbati S and Citterio S (2007) Thiol-peptide level and proteomic changes in response to cadmium toxicity in *Oryza sativa* L. roots. Environmental and Experimental Botany, **59**, 381 – 392.

∴ Alcántara E, Romera FJ, Cañete M and De La Guardia MD (1994) Effects of heavy metals on both induction and function of root Fe(III)reductase in Fe-deficient cucumber (*Cucumis sativus* L.) plants. Journal of Experimental Botany, **45**, 1893 – 1898.

∴ Aloui A, Recorbet G, Gollotte A, Robert F, Valot B, Gianinazzi-Pearson V, Aschi-Smiti S and Dumas-Gaudot E (2009) On the mechanisms of Cd stress alleviation in *Medicago truncatula* by arbuscular mycorrhizal symbiosis: a root proteomic study. Proteomics, **9**, 420 – 433.

∴ Alvarez S, Berla BM, Sheffield J, Cahoon RE, Jez JM and Hicks LM, 2009, Comprehensive analysis of the *Brassica juncea* root proteome in response to cadmium exposure by complementary proteomic approaches. Proteomics, **9**, 2419 – 2431.

∴ Andria V, Reichenauer TG and Sessitsch A (2009) Expression of alkane monooxygenase (alkB) genes by plant-associated bacteria in the rhizosphere and endosphere of Italian ryegrass (*Lolium multiflorum* L.) grown in diesel contaminated soil. Environmental Pollution, **157**, 12: 3347 – 3350.

∴ Anke M, Groppel B and Schmidt A (1987) New results on the essentiality of Cd in ruminants. Trace substances in environmental health : proceedings of the University of Missouri's annual conference on Trace Substances in Environmental Health, **21**, 556 – 566.

∴ Antonovics J, Bradshaw AD and Turner RG (1971) Heavy metal tolerance in plants, Advances in Environmental Science and Technology, **7**, 1 – 85.

∴ Arshad M, Saleem M and Hussain S (2007) Perspectives of bacterial ACC demainase in phytoremediation. TRENDS in Biotechnology, **25**, 356 – 362.

∴ Arteca RN and Arteca JM (2007) Heavy-metal-induced ethylene production in Arabidopsis thaliana. *Journal of Plant Physiology*, **164**, 1480 – 1488.

∴ Ashan N, Renaut J and Komatsu S (2009) Recent developments in the application of proteomics to the analysis of plant responses to heavy metals. Proteomics, **9**, 2602 – 2621.

∴ Ashan N, Nakamura T and Komatsu S (2012) Differential responses of microsomal proteins and metabolites in two contrasting cadmium (Cd)-accumulating soybean cultivars under Cd stress. Amino Acids, **42**, 317 – 327.

∴ Axelsen K and Palmgren M (2001) Inventory of the superfamily of P-type ion pumps in Arabidopsis, Plant Physiology, **126**, 696 – 706.

∴ Bah AM, Sun H, Zhou J, Dai H, Zhang G and Wu F (2010) Comparative proteomic analysis of *Typha angustifolia* leaf under chromium, cadmium and lead stress. Journal of Hazardous Materials, **184**, 191 – 203.

∴ Baker AJM, Ewart K, Hendry GAF, Thorpe PC and Walker PL (1990) The evolutionary basis of Cd tolerance in higher plants. In: 4th International Conference on Environmental Contamination, Barcelona, pp. 23 – 29.

∴ Baldin MA (2003) Protein identification by mass spectrometry. Molecular and Cellular Proteomics, **3**, 1 – 9.

∴ Balgley BM, Laudeman T, Yang L, Song T and Lee CS (2007) Comparative evalutation of tandem MS search algorithms using a target-decoy search strategy. Molecular and Cellular Proteomics, **6**, 1599 - 1608.

∴ Banerji S and Flieger A (2004) Patatin-like proteins: a new family of lipolytic enzymes present in bacteria? *Microbiology*, **150**, 522 – 525.

∴ Barac T, Taghavi S, Borremans B, Provoost A, Oeyen L, Colpaert JV, Vangronsveld J and van der Lelie D (2004) Enineered endophytic bacteria improve phytoremediation of water-soluble, volatile, organic pollutants. Nature Biotechnology, **22**, 583 – 588.

∴ Barber RD and Donohue TJ (1998) Pathways for transcriptional activation of a glutathione-dependent formaldehyde dehydrogenase gene. *Journal of Molecular Biology*, **280**, 775 – 784.

∴ Barrett J, Brophy PM and Hamilton JV (2005) Analysing proteomic data. International Journal Parasitology*,* **35**, 543 – 553.

∴ Belimov AA, Hontzeas N,Safronoav VI,Demchinskaya SV, Pilluzza G, Bullitta S and Glick BR (2005) Cd-tolerant plant growth-promoting bacteria

associated with the roots of Indian mustard (*Brassica juncea* L. Czern.). Soil Biology and Biochemistry, **37**, 241 – 250.

∴ Benavides MP, Gallego SM and Tomaro ML (2005) Cd toxicity in plants. Brazilian Journal of Plant Physiology, **17**, 21 – 34.

∴ Bennett LE, Burkhead JL, Hale KL, Terry N, Pilon M and Pilon-Smits EHA (2003) Analysis of transgenic Indian mustard plants for phytoremediation of metal-contaminated mine tailings. Journal of Environmental Quality, **32**, 432 – 440.

∴ Berlett BS and Stadtman ER (1997) Protein oxidation in aging, diseases, and oxidative stress. Journal of Biological Chemistry, **272**, 20313 – 20316.

∴ Bi Y, Chen W, Zhang W, Zhou Q, Yun L and Xing D (2009) Production of reactive oxygen species, impairment of photosynthetic function and dynamic changes in mitochondria are early events in cadmium-induced cell death in Arabidopsis. *Biology of the Cell*, **101**, 629 – 643.

∴ Bingham FT, Sposito G and Strong JE (1984) The effect of chloride on the availability of cadmium. Journal of Environmental Quality, **13**, 71 – 74.

∴ Binz PA, Barkovich R, Beavis RC, Creasy D, Horn DM, Julian RK Jr, Seymour SL, Taylor CF and Vandenbrouck Y (2008) Guidelines for reporting the use of mass spectrometry informatics in proteomics. Nature Biotechnology, **26**, 862.

∴ Block CC, Hill JH and McGee DC (1998) Seed transmission of Pantoea stewartii in field and sweet corn. Plant Disease, **82**, 775 – 780.

∴ Bohler S, Bagard M, Oufir M, Planchon S, Hoffmann L, Jolivet Y, Hausman JF, Dizengremel P and Renaut J (2007) A DIGE analysis of developing poplar leaves subjected to ozone reveals major changes in carbon metabolism. Proteomics, **7**, 1584 – 1599.

∴ Braud A, Jézéquel K, Bazot S and Lebeau T (2009) Enhanced phytoextraction of an agricultural Cr- and Pb-contaminated soil by bioaugmentation with siderophore-producing bacteria. Chemosphere, **74**, 280 – 286.

∴ Brown S, Chaney R, Angle J and Baker A (1994) Phytoremediation potential of *Thlaspi caerulescens* and bladder campion for zinc and Cd-contaminated soil. Journal of Environmental Quality, **23**, 1151 – 1157.

∴ Bruins MR, Kapil S and Oehme FW (2000) Microbial resistance to metals in the enviroment. Ecotoxicology and Environmental Safety, **45**, 198 – 207.

∴ Cai Y, Cao F, Wei K, Zhang G and Wu F (2011) Genotypic dependent effect of exogenous glutathione on Cd-induced changes in proteins, ultrastructure

and antioxidant defence enzymes in rice seedlings. Journal of Hazardous Materials, **192**, 1056 – 1066.

∴ Cairns DA, Thompson D, Perkins DN, Stanley AJ, Selby PJ and Banks RE (2008) Proteomic profiling using mass spectrometry – does normalising by total ion current potentially mask some biological differences? Proteomics, **8**, 21 – 27.

∴ Callahan DL, Baker AJM, Kolev SD and Wedd AG (2006) Metal ion ligands in hyperaccumulating plants. Journal of Biological Inorganic Chemistry, **11**, 2 – 12.

∴ Carpentier SC, Witters E, Deckers P, Swennen R and Panis B (2005) Preparation of protein extracts from recalcitrant plant tissues: An evaluation of different methods for two-dimensional gel electrophoresis analysis. Proteomics, **5**, 2497 – 2507.

∴ Carpentier SC, Panis B, Swennen R, Lammertyn J, and Vlahou A, (2007) Finding the significant markers: statistical analysis of proteomic data. In: Vlahou, A (ed.) Methods in Molecular Biology. Humana Press Inc., Totowa, NJ, pp. 327 – 347.

∴ Cataldo CD, Garland TR and Widung RE (1983) Cd uptake, kinatics in intact soybean plants. Plant Physiology, **73**, 844 – 848.

∴ Chaney RL (1983) Plant uptake of inorganic waste constituents. In: Parr JF, Marsh PB, Kla JM (eds.) Land Exposure of Hazardous Waste. Noyes Data Corporation, Parkridge, USA, pp. 50 – 76.

∴ Chen L, Shinde U, Ortolan TG and Madura K (2001) Ubiquitin-associated domains in rad23 bind ubiquitin and promote multi-ubiquitin chain assembly. *EMBO Reports*, **2**, 933 – 938.

∴ Chen J, Tsai A, O'Leary SE, Petrov A and Puglisi JD (2012) Unraveling the dynamics of ribosome translocation. *Current Opinion in Structural Biology*, **22**, 804 – 814.

∴ Chen ZJ, Sheng XF, He LY, Huang Z and Zhang WH (2013) Effects of root inoculation with bacteria on the growth, Cd uptake and bacterial communities associated with rape grown in Cd-contaminated soil. *Journal of Hazardous Materials*, **15**, 244 – 245.

∴ Cho SH, Hoang QT, Kim YY, Shin HY, Ok SH, Bae JM and Shin JS (2006) Proteome analysis of gametophores identified a metallothionein involved in various abiotic stress responses in *Physcomitrella patens*. Plant Cell Reports, **25**, 475 – 488.

∴ Cilia M, Fish T, Yang X, McLaughlin M, Thannhauser TW and Gray S (2009) A comparison of protein extraction methods suitable for gel-based

proteomic studies of aphid proteins. Journal of Biomolecular Techniques, **20**, 201 – 215.

∴ Clarke DJ, Mondesert G, Segal M, Bertolaet BL, Jensen S, Wolff M, Henze M and Reed SI (2001) Dosage suppressiors of *pds1* implicate ubiquitin-associated domains in checkpoint control. *Molecular and Cellular Biology*, **21**, 1997 – 2007.

∴ Clarck BN and Gutstein HB (2008) The myth of automated, high-throughput two-dimensional gel analysis. Proteomics, **8**, 1197 – 1203.

∴ Clejan LA and Cederbaum AI (1993) Stimulation by paraquat of microsomal and cytochrome P450-dependent oxidation of glycerol to formaldehyde. *Biochemical Journal*, **295**, 781 – 786.

∴ Clemens S, Palmgreen MG and Kramer U (2002) A long way ahead: understanding and engineering plant metal accumulation. Trends in Plant Science, **7**, 309 – 315.

∴ Clemens S (2006a) Evolution and function of phytochelatin synthases. Journal of Plant Physiology, **163**, 319 – 332.

∴ Clemens S (2006b) Toxic metal accumulation, responses to exposure and mechanisms of tolerance in plants. Biochimie, **88**, 1707 – 1719.

∴ Clifton J, Huang F, Rucevic M, Cao L, Hixson D and Josic D (2011) Protease inhibitors as possible pitfalls in proteomic analyses of complex biological samples. *Journal of Proteomics*, **74**, 935 – 941.
doi: 10.1016/j.jprot.2011.02.010

∴ Cobbett CS (2000) Phytochelatin biosynthesis and function in heavy metal detoxification. Current Opinion in Plant Biology, **3**, 211 – 216.

∴ Cobbett C and Goldsbrough P (2002) Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annual Review of Plant Biology, **53**, 159 – 82.

∴ Compant S, Clément C and Sessitsch A (2010) Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. Soil Biology and Biochemistry, **42**, 669 – 678.

∴ Cosio C, DeSantis L, Frey B, Diallo S and Keller C (2005) Distribution of Cd in leaves of *Thlaspi caerulescens*. Journal of Experimental Botany, **56**, 412: 765 – 775.

∴ Cummins I, McAuley K, Fordham-Skelton A, Schwoerer R, Steel PG, Davis BG and Edwards R (2006) Unique regulation of the active site of the serine esterare S-formylglutathione hydrolase. *Journal of Molecular Biology*, **359**, 422 – 432.

∴ Cunningham JE and Kuiack C (1992) Production of citric and oxalic acids and solubilization of Cd phosphate by *Penicilium Bilaii*. Environmental Microbiology, **58**, 1451 – 1458.

∴ Cuypers A, Smeets K, Vangronsveld J (2009) Heavy metal stress in plants. In: Hirt H (ed.) Plant stress biology: from Genomics to system biology. Wiley – VCH Verslag, Weinheim, Baden-Württemberg, Germany, pp. 161 – 178.

∴ Cuypers A, Plusquin M, Remans T, Jozefczak M, Keunen E, Gielen H, Opdenakker K, Nair AR, Munters E, Artois TJ, Nawrot T, Vangronsveld J and Smeets K (2010) Cd stress: an oxidative challenge. Biometals, **23**, 927 – 940.

∴ Cuypers A, Smeets K, Ruytinx J, Opdenakker K, Keunen E, Remans T, Horemans N, Vanhoudt N, Van Sanden S, Van Belleghem F, Guisez Y, Colpaert J and Vangronsveld J (2011) The cellular redox state as a modulator in cadmium and copper responses in *Arabidopsis thaliana* seedlings. Journal of Plant Physiology, **168**, 309 – 316.

∴ Cuypers A, Keunen E, Bohler S, Jozefczak M, Opdenakker K, Gielen H, Vercampt H, Bielen A, Schellingen K, Vangronsveld J, Remans T (2012). In: Metal Toxicity in Plants: Perception, Signalling and Remediation (eds. D.K.G. Gupta & L.M. Sandalio) Cadmium and copper stress induce a cellular oxidative challenge leading to damage versus signalling. Springer-Verlag GmbH Berlin Heidelberg, pp.65-90.

∴ Dalcorso G, Farinati S, Maistri S and Furini A (2008) How plants cope with Cd: staking all on metabolism and gene expression. Journal of Integrative Plant Biology, **50**, 10: 1268 – 1280.

∴ DalCorso G, Farinati S and Furini A (2010) Regulatory networks of Cd stress in plants. Plant Signaling and Behavior, **5**, 6: 663 – 667.

∴ Deckert J (2005) Cd toxicity in plants: Is there any analogy to its carcinogenic effect in mammalian cells? BioMetals, **18**, 475 – 481.

∴ Degroeve S, Colaert N, Vandekerckhove J, Gevaert K and Martens L, 2011, A reproducibility-based evaluation procedure for quantifying the differences between MS/MS peak intensity normalization methods. Proteomics, **11**, 1172 – 1180.

∴ Delhaize EP and Ryan R (1995) Aluminium toxicity and tolerance in plants. Plant Physiology, **107**, 315 – 321.

∴ Denecke J, Goldman MHS, Seurinck J and Botterman J (1991) The tobacco luminal binding protein is encoded by a multigene family. *The Plant Cell*, **3**, 1025 – 1035.

∴ Dhondt S, Geoffroy P, Stelmach BA, Legrand M and Heitz T (2000) Soluble phospholipase A2 activity is induced before oxylipin accumulation in tobacco mosaic virus-infected tobacco leaves and is contributed by patatin-like enzymes. *Plant Journal*, **23**, 431 – 440.

∴ Di Baccio D, Tognetti R, Sebastiani L and Vitagliano C (2003) Responses of *Populus deltoides* x *Populus nigra* (*Populus* x *euramericana*) clone I-214 to high zinc concentrations. New Phytologist, **159**, 443 – 452.

∴ Dickinson NM, Baker AJM, Doronila A, Laidlaw S and Reeves RD (2009) Phtyoremediation of inorganics: realism and synergies. International Journal of Phytoremediation, **11**, 2: 97 – 114.

∴ Diels L, Van Roy S, Taghavi S, Doyen W, Leysen R and Mergeay M (1993) The use of bacteria immobilized in a tubular membrane reactor for heavy metal recovery and degradation of chlorinate aromatics. Journal of Membrange Science, **100**, 249 – 258.

∴ Dietz KJ, Baier M and Kramer U (1999) Free radicals and reactive oxygen species as mediators of heavy metal toxicity in plants. In: Prasad MNV, Hagemeyer J (eds.) Heavy metal stress in plants: from molecules to ecosystems. Springer-Verlag, Berlin, Germany, pp. 73 – 97.

∴ do Nascimento CWA and Xing B (2006) Phytoextraction: a review on enhanced metal availability and plant accumulation. Scientia Agricola, **63**, 299 – 311.

∴ Doner HE (1978) Chloride as a factor of mobilities of Ni (II), Cu (II), Cd (II) in soil. Soil Science Society of America Journal, **42**, 882 – 885.

∴ Doxy AC, Yaish MWF, Moffatt BA, Griffith M and McConkey BJ (2007) Functional divergence in the Arabidopsis B-1.3-glucanase gene family inferred by phylogenetic reconstruction of expression states. *Molecular Biology and Evolution*, **24**, 1045 – 1055.

∴ Doyle JJ, Marshal RT and Pfander WH (1975) Effects of cadmium on the growth and uptake of microorganisms. *Applied and Environmental Microbiology*, **29**, 562 – 564.

∴ Dubos C and Plomion C (2001) Drought differentially affects expression of a PR-10 protein, in needles of maritime pine (Pinus pinaster Ait.) seedlings. *Journal of Experimental Botany*, **52**, 1143 – 1144.

∴ Duff SMG, Sarath G and Plaxton WC (1994) The role of acid phosphatises in plant phosphorus metabolism. Physiologia Plantarum, **90**, 791 – 800.

∴ Dumont D, Noben JP, Raus J, Stinissen P and Robben J (2004) Proteomic analysis of cerebrospinal fluid from multiple sclerosis patients. Proteomics, **4**, 2117 – 2124.

∴ Durand TC, Sergeant K, Planchon S, Carpin S, Label P, Morabito D, Hausman JF and Renaut J (2010) Acute metal stress in *Populus tremula x P. Alba* (717-1B4 genotype): Leaf and cambial proteome changes induces by cadmium^{2+}. Proteomics, **10**, 349 – 368.

∴ Eapen S and D'Souza SF (2005) Prospects of genetic engineering of plants for phytoremediation of toxic metals. Biotechnology Advances, **23**, 97 – 114.

∴ Espagne C, Martinez A, Valot B, Meinnel T and Giglione C (2007) Alternarive and effective proteomic analysis in Arabidopsis. Proteomics, **7**, 3788 – 3799.

∴ Evanglou MW, Ebel M and Schaeffer A (2007) Chelate assisted phytoextraction of heavy metals from soil. Effect, mechanism, toxicity and fate of chelating agents. Chemosphere, **68**, 989 – 1003.

∴ Evangelou MW, Deram A, Gogos A, Studer B and Schulin R (2012) Assessment of suitability of tree species for the production of biomass on trace element contaminated soils. *Journal of Hazardous Material*, **209-210**, 233 – 239.

∴ Fall R and Benson AA (1996) Leaf methanol: the simplest natural product from plants. *Trends in Plant Science*,**1**, 296 – 301.

∴ Farinati S, DalCorso G, Bona E, Corbella M, Lampis S, Cecconi D, Polati R, Berta G, Vallini G and Furini A (2009) Proteomic analysis of *Arabidopsis halleri* shoots in response to the heavy metals Cd and zinc and rhizosphere microorganisms. Proteomics, **9**, 4837 – 4850.

∴ Fagioni M and Zolla L (2009a) Does the different proteomic profile found in apical and basal leaves of spinach reveal a strategy of this plant toward cadmium pollution response? Journal of Proteome Research, **8**, 2519 – 2529.

∴ Fagioni M, D'Amici GM, Timperio AM and Zolla L (2009b) Proteomic analysis of multiprotein complexes in the thylakoid membrane upon cadmium treatment. Journal of Proteome Research, **8**, 310 – 326.

∴ Farinati S, DalCorso G, Bona E, Corbella M, Lampis S, Cecconi D, Polati R, Berta G, Vallini G and Furini A (2009) Proteomic analysis of Arabidopsis halleri shoots in response to the heavy metals cadmium and zinc and rhizosphere microorganisms. Proteomics, **9**, 4837 – 4850.

∴ Fernández AP and strand A (2008) Retrograde signalling and plant stress: plastid signals initiate cellular stress responses. *Current Opinion in Plant Biology*, **11**, 509 – 513.

∴ Ferry JG (1990) Formate dehydrogenase. *FEMS Microbiology Reviews*, **87**, 377 – 382.

∴ Filipic M (2012) Mechanisms of cadmium-induced genomic instability. *Mutation Research*, **733**, 69 – 77.

∴ Flores HE, Dai Y, Cuello JL, Maldonado-Mendoza IE and Loyola-Vargas (1993) Green roots: photosynthesis and photoautothropy in an underground plant organ. *Plant Physiology*, **101**, 363 – 371.

∴ Fojtova M, Fulneckova J, Fajkus J and Kovarik A (2002) Recovery of tobacco cells from Cd stress is accompanied by DNA repair and increased telomerase activity. Journal of Experimental Botany, **378**, 2151 – 2158.

∴ Freist W and Gauss DH (1995) Lysyl-tRNA synthetase. *Biological Chemistry Hoppe-Seyler*, **376**, 451 – 472.

∴ Fu A, He Z, Cho HS, Lima A, Buchanan BB and Luan S (2007) A chloroplast cyclophilin functions in the assembly and maintenance of photosystem II in *Arabidopsis thaliana*. *PNAS*, **104**, 15947 – 15952.

∴ Fujita Y, Robroek BJM, de Ruiter PC, Heil GW and Wassen MJ (2010) Increased N affects P uptake of eight grassland species: the role of root surface posphatase activity. Oikos, **119**, 1665 – 1673.

∴ Fusco N, Micheletto L, Dal Corso G, Borgato L and Furini A (2005) Identification of Cd-regulated genes by cDNA-AFLP in the heavy metal accumulator Brassica juncea L. Journal of Experimental Botany, **56**, 3017 – 3027.

∴ Gallego EM, Pena LB, Barcia RA, Azpilicueta CE, Iannone MF, Rosales EP, Zawoznik MS, Groppa MD and Benavides MP (2012) Unravelling cadmium toxicity and tolerance in plants: insight into regulatory mechanisms. *Journal of environmental and experimental botany*, **83**, 33 – 46.

∴ Garavelli JS (2004) The RESID database of protein modifications as a resource and annotation tool. Proteomics, **4**, 1527 – 1533.

∴ Garcia-Hernandez M, Murphy A and Taiz L (1998) Metallothioneins 1 and 2 have distinct but overlapping expression patterns in Arabidopsis. Plant Physiology, **118**, 387 – 397.

∴ Garg G and Kataria SK, Phytoremediation potential of *Raphanus sativus* (L.), *Brassica juncea* (L.) and *Triticum aestivum* (L.) for copper contaminated soil. Proceedings of the 53rd Annual Meeting of the International Society for the Systems Sciences, (AMISSS`10), Gautam Buddha University, India, pp. 1-10

∴ Ge C, Ding Y, Wang Z, Wan D, Wang Y, Shang Q and Luo S (2009) Responses of wheat seedlings to cadmium, mercury and trichlorobenze stresses. Journal of Environmental Sciences, **21**, 806 – 813.

∴ Ghoshroy S, Freedman K, Lartey R and Citovsky V (1998) Inhibition of plant viral systemic infection by non-toxic concentrations of Cd. Plant Journal, **13**, 591 – 602.

∴ Giachetti G and Sebastiani L (2006) Metal accumulation in poplar plant grown with industrial wastes. Chemosphere, **64**, 446 – 454.

∴ Gianazza E, Wait R, Sozzi A, Regondi S, Saco D, Labra M and Agradi E (2007) Growth and protein profile changes in *Lepidium sativum* L. plantlets exposed to cadmium. Environmental and Experimental Botany, **59**, 179 – 187.

∴ Gibson F, Anderson L, Babnigg G, Baker M, Berth M, Binz PA, Borthwick A, Cash P, Day BW, Friedman DB, Garland D, Gutstein HB, Hoogland C, Jones NA, Khan A, Klose J, Lamond AI, Lemkin PF, Lilley KS, Minden J, Morris NJ, Paton NW, Pisano MR, Prime JE, Rabilloud T, Stead DA, Taylor CF, Voshol H, Wipat A and Jones AR (2008) Guidelines for reporting the use of gel electrophoresis in proteomics. Nature Biotechnology, **26**, 863 - 864.

∴ Gill SS and Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry*, **48**, 909 – 930.

∴ Gill SS and Tuteja N (2011) Cd stress tolerance in crop plants. Plant Signaling and Behavior, **6**, 2: 215 – 222.

∴ Gillet S, Decottignies P, Chardonnet S and Le Maréchal P (2006) Cadmium response and redoxin targets in *Chlamydomonas reinhardtii*: a proteomics approach. Phytosynthesis Research, **89**, 201 – 211.

∴ Gilbert HF (2011) Protein disulfide isomerase. *eLS*

∴ Giordano PM, Behel AD, Lawrence JE, Solleau JM and Broadford BN (1983) Mobility in soil and plant availability of metals derived from incinerated municipal refuse. Environmental Science and Technology, **17**, 192 – 198.

∴ Glick BR (2003) Phytoremediation: synergistic use of plants and bacteria to clean up the environment. Biotechnology Advances, **21**, 383 – 393.

∴ Glick BR (2005) Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. FEMS Microbiology Letters, **251**, 1 – 7.

∴ Glick BR (2010) Using soil bacteria to facilitate phytoremediation. Biotechnology Advances, **28**, 367 – 374.

∴ Gobe G and Crane D (2010) Mitochondria, reactive oxygen species and Cd toxicity in the kidney. Toxicology Letters, **198**, 1: 49 – 55.

∴ Godt J, Scheidig F, Grosse-Siestrup C, Esche V, Brandenburg P, Reich A and Groneberg DA (2006) The toxicity of cadmium and resulting hazards for

human health. *Journal of Occupational Medicine and Toxicology*, **1**, 22. doi: 10.1186/1745-6673-1-22

∴ Gong JM, Lee DA and Schroeder JI (2003) Long-distance root-to-shoot transport of phytochelatins and Cd in Arabidopsis, Proceedings of the National Academy of Science of the USA, **100**, 10118 – 10123.

∴ Görg A, Weiss W and Dunn MJ (2004) Current two-dimensional electrophoresis technology for proteomics. Proteomics, **4**, 3665 – 3685.

∴ Grant CA, Buckley WT, Bailey LD and Selles F (1998) Cd accumulation in crops. Canadian Journal of Plant Science, **78**, 1 – 17.

∴ Grant CA, Clarcke JM, Duguid S and Chaney RL (2008) Selection and breeding of plant cultivars to minimize Cd accumulation. Science of the Total Environment, **390**, 301 – 310.

∴ Grill E, Winnacker EL and Zenk MH (1985) Phytochelatins: the principal heavy-metal complexing peptides of higher plants. Science, **230**, 674 – 676.

∴ Grill E, Winnacker EL and Zenk MH (1987) Phytochelatins, a class of heavy metal-binding peptides from plants are functionally analogous to metallothioneins. Proceedings of the National Academy of Science of the USA, **84**, 439 – 443.

∴ Groβe C, Grass G, Anton A, Franke S, Santos AN, Lawley B, Brown NL and Nies DH (1999) Transcriptional organization of the *czc* heavy metal homeostasis determinant from *Alcaligenes eutrophus*. Journal of Bacteriology, **181**, 8: 2385 – 2393.

∴ Groβe C, Anton A, Hoffmann T, Franke S, Schleuder G and Nies DH (2004) Identification of a rehulatory pathway that controls the heavy metal resistance system Czc via promoter *czcNp* in *Ralstonia metallidurans*. Archives of Microbiology, **182**, 109 – 118.

∴ Gustafsson JS, Ceasar R, Glasbey CA, Blomberg A and Rudemo M (2004) Statistical exploration of variation in quantitative two-dimensional gel electrophoresis. *Proteomics*, **4**, 3791 – 3799.

∴ Guzder SN, Bailly V, Sung P, Prakash L and Prakash S (1995) Yeast DNA repair protein rad23 promotes complex formation between transcription factor TFIIH and DNA damage recognition factor RAD14. *Journal of Biological Chemistry*, **270**, 8385 – 8388.

∴ Hajduch M, Rakwal R, Agrawal GK, Yonekura M and Pretova A (2001) High-resolution two-dimensional electrophoresis separation of proteins from metal-stressed rice (Oryza sativa L.) leaves: Drastic reductions/fragmentation of ribulose-1,5-bisphosphate

carboxylase/oxygenase and induction of stress-related proteins. Electrophoresis, **22**, 2824 – 2831.

∴ Hall JL (2002) Cellular mechanisms for heavy metal detoxification and tolerance. Journal of Experimental Botany, **53**, 366: 1 – 11.

∴ Halliwell B (2006) Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiology, **141**, 312 – 322.

∴ Hamer DH (1986) Metallothioneins. Annual Review of Biochemistry, **55**, 913 – 951.

∴ Hanikenne M, Talke IN, Haydon MJ, Lanz C, Nolte A, Motte P Kroymann J, Weigel D and Kramer U (2008) Evolution of metal hyperaccumulation required cis-regulatory changes and triplication of HMA4. Nature, **453**, 391 – 395.

∴ Hartwig A (2010) Mechanisms in Cd-induced carcinogenicity: recent insights. BioMetals, **23**, 951 – 960.

∴ Hasan SA, Fariduddin Q, Ali B, Hayat S and Ahmad A (2009) Cd: toxicity and tolerance in plants. Journal of Environmental Biology, **30**, 2: 165 – 174.

∴ Hassan M-e-T, van der Lelie D, Springael D, Römling U, Ahmed N and Mergeay M (1999) Identification of a gene cluster, *czr*, involved in Cd and zinc resistance in *Pseudomonas aeruginosa*. Gene, **238**, 417 – 425.

∴ Haslam R, Rust S, Pallett K, Cole D and Coleman J (2002) Cloning and characterisation of S-formylglutathione hydrolase from *Arabidopsis thaliana*: a pathway for formaldehyde detoxification. Plant physiology and Biochemistry, **40**, 281 – 288.

∴ Heber U, Wiese C, Neimanis S, Savchenko G, Bukhov NG and Hedrich R (2002) Energy-dependent solute transport from the apoplast into the symplast of leaves during transpiration. Russian Journal of Plant Physiology, **49,** 32 – 43.

∴ Heide H, Kalisz HM and Follmann H (2004) The oxygen evolving enhancer protein 1 (OEE1) of photosystem II in green algae exhibits thioredoxin activity. *Journal of Plant Physiology*, **161**, 139 – 149.

∴ Hensel LL, Grbic V, Baumgarten DA and Bleecker AB (1993) Developmental and age-related processes that influence the longevity and senescence of photosynthetic tissues in Arabidopsis. *The Plant Cell*, **5**, 553 – 564.

∴ Helsens K, Timmerman E, Vandekerckhove J, Gevaert K and Martens L (2008) Peptizer, a tool for assessing false positive peptide identifications and manually validating selected results. Molecular and Cellular Proteomics, **12**, 2364 - 2372.

∴ Hernandez LE, Carpena-Ruiz R and Garate A (1996) Alterations in the mineral nutrition of pea seedlings exposed to Cd. Journal of Plant Nutrition, **19**, 1581 – 1598.

∴ Higaki T, Sano T and Hasezawa S (2007) Actin microfilament dynamics and actin side-binding proteins in plants. Current Opinion in Plant Biology, **10**, 549 – 556.

∴ Hirt H, Casari G and Barta A (1989) Cd enhanced gene expression in suspension-culture cells of tobacco. Planta, **179**, 414 – 420.

∴ Hoagland DR and Arnon DI (1938) The water culture method for growing plants without soil. California Agricultural Experiment Station Bulletin, **347**, 36 – 39.

∴ Hoagland C, O'Gorman M, Bogard P, Gibson F, Berth M, Cockell SJ, Ekefjärd A, Forsstrom-Olsson O, Kapferer A, Nilsson M, Martínez-Bartolomé S, Albar JP, Echevarría-Zomeño S, Martínez-Gomariz M, Joets J, Binz PA, Taylor CF, Dowsey A and Jones AR (2010) Minimum Information About a Proteomics Experiment (MIAPE). Guidelines for reporting the use of gel image informatics in proteomics. Nature Biotechnology, **28**, 655 - 656.

∴ Van der Honing HS, Kieft H, Emons AMC and Ketelaar T (2012) Arabidopsis VILLIN2 and VILLIN3 are required for the generation of thick actin filament bundles and for directional organ growth. *Plant Physiology*, **158**, 1426 – 1438.

∴ Hopkins MT, Lampi Y, Wang T-W, Liu Z and Thompson JE (2008) Eukaryotic translation initiation factor 5a is involved in pathogen-induced cell death and development of disease symptoms in Arabidopsis. *Plant Physiology*, **148**, 479 – 489.

∴ Horemans N, Raeymaekers T, Van Beek K, Nowocin A, Blust R, Broos K, Cuypers A, Vangronsveld J and Guisez Y (2007) Dehydroascorbate uptake is impaired in the early response of *Arabidopsis* plant cell cultures to cadmium. *Journal of Experimental Botany*, **58**, 4307 – 4317.

∴ Horky D, Illek J and Pechova A (2002) Histochemical and ultrahistochemical localization of heavy metals in calf organs. Microscopy Research and Technique, **56**, 435 – 450.

∴ Hradilová J, Rehulka P, Rehulkova H, Vrbová M, Griga M and Brzobohatý (2010) Comparative analysis of proteomics changes in contrasting flax cultivars upon cadmium exposure. Electrophoresis, **31**, 421 – 431.

∴ Huff J, Lunn RM, Waalkes MP, Tomatis L and Infante PF (2007) Cadmium-induced cancers in animals and humans. *International Journal of Occupational and Environmental Health*, **13**, 202 – 212.

∴ Husebye H, Chadchawan S, Winge P, Thangstad OP and Bones AM (2002) Guard cell- and phloem idioblast-specific expression of thioglucoside glucohydrolase 1 (myrosinase) in Arabidopsis. *Plant Physiology*, **128**, 1180 – 1188.

∴ Iakimova ET, Woltering EJ, Kapchina-Toteva VM, Harren FJ and Cristescu SM (2008) Cd toxicity in cultured tomato cells: role of ethylene, proteases and oxidative stress in cell death signaling. Cell Biology International, **32**, 12: 1521 – 1529.

∴ Jackson JE (2003). A user's guide to principal components. John Wiley and Sons, Inc., NY, USA. pp. 563.

∴ Jarup L (2003) Hazards of heavy metal contamination. British Medical Bulletin, **68**, 167 – 182.

∴ Jarup L, Berglund M, Elinder GG, Nordberg G and Vahter M (1998) Health effects of cadmium exposure. Scandinavian Journal Work Environmental Health, **24,** 1 - 51.

∴ Johri N, Jacquillet G and Unwin R (2010) Heavy metal poisoning: the effects of Cd on the kidney. Biometals, **23**, 5: 783 – 792.

∴ Jomova K and Valko M (2011) Advances in metal-induced oxidative stress and human disease. Toxicology, **283**, 65 – 87.

∴ Jones AR, Siepen JA, Hubbard SJ and Paton NW (2009) Improving senstiticity in proteome studies by analysis of false discovery rates for multiple search engines. Proteomics, **8**, 1220 – 1229.

∴ Jones AR, Carroll K, Knight D, Maclellan K, Domann PJ, Legido-Quigley C, Huang L, Smallshaw L, Mirzaei H, Shofstahl J and Paton NW (2010) Minimum Information About a Proteomics Experiment (MIAPE). Guidelines for reporting the use of column chromatography in proteomics. Nature Biotechnology, **28**, 654.

∴ Jonscher KR (2005) Validating sequence assignments for peptide fragmentation patterns: A primer in MS/MS sequence identification. www.proteomesoftware.com

∴ Jorrín-Novo J, Maldonado AM, Echevarría-Zomeño S, Valledor L, Castillejo MA, Curto M, Valero J, Sghaier B, Donoso G and Redondo I (2009) Plant proteomics update (2007 – 2008): Second-generation proteomic techniques, an appropriate experimental design and data analysis to fulfil MIAPE standards, increase plant proteome coverage and expand biological knowledge. Journal of Proteomics, **72**, 285 – 314.

∴ Jozefczak M, Remans T, Vangronsveld J and Cuypers A (2012) Glutathione is a key player in metal-induced oxidative stress defences. *International Journal of Molecular Sciences*, **13**, 3145 – 3175.

∴ Kacperska A (2004) Sensor types in signal transduction pathways in plant cells responding to abiotic stressors: do they depend on stress intensity? Physiologia Plantarum, **122**, 159 – 68.

∴ Karp NA and Lilley KS (2007) Design and analysis issues in quantitative proteomics studies. *Proteomics*, **7**, 42 – 50.

∴ Kaschl A, Römheld V and Chen Y (2002) Cadmium binding by fractions of dissolved organic matter and humic substances from municipal solid waste compost. Journal of Environmental Quality, **31**, 1885 – 1892.

∴ Kask L, Larsson K and Bjellqvist B (2009) Elimination of basic gaps at high pH values in 2-DE. Proteomics, **9**, 5558 – 5561.

∴ Keller A, Nesvizhskii AI, Kolker E and Aebersold R (2002) Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. Analytical Chemistry, **74**, 5383 – 5392.

∴ Kendrick K G and Margolis J (1970) Isoelectric focusing and gradiente gel electrophoresis gel. A 2-dimensional technique. Analitical Biochemistry, **33**, 204 – 207.

∴ Kerk D, Bulgrien J, Smith DW and Gribskov M (2003) Arabidopsis proteins containing similarity to the universal stress protein domain of bacteria. *Plant Physiology*, **131**, 1209 – 1219.

∴ Ketelaar T, Faivre-Moskalenko C, Esseling JJ, de Ruijter NC, Grierson CS, Dogterom M and Emons AM (2002) Positioning of nuclei in Arabidopsis root hairs: an actin-regulated process of tip growth. *Plant Cell*, **14**, 2941 – 2955.

∴ Keunen E, Remans T, Bohler S, Vangronsveld J and Cuypers A (2011) Metal-induced oxidative stress and plant mitochondria. International Journal of Molecular Sciences, **12**, 6894 – 6918.

∴ Keunen E, Truyens S, Bruckers L, Remans T, Vangronsveld J, Cuypers A (2011) Survival of Cd-exposed *Arabidopsis thaliana*: Are these plants reproductively challenged? Plant Physiology and biochemistry 49: 1084 – 1091.

∴ Kieffer P, Dommes J,Hoffmann L, Hausman JF and Renaut J (2008) Quantitative changes in protein expression of Cd-exposed poplar plants. Proteomics, **8**, 2514 – 2530.

∴ Kieffer P, Planchon S, Oufir M, Ziebel J, Dommes J, Hoffmann L, Hausman JF and Renaut J (2009a) Combining proteomics and metabolite analyses to

unravel Cd stress-response in poplar leaves. Journal of Proteome Research, **8**, 400 – 417.

∴ Kieffer P, Schröder P, Dommes J, Hoffmann L, Renaut J and Hausman JF (2009b) Proteomic and enzymatic response of poplar Cd stress. Journal of Proteomics, **72**, 379 – 396.

∴ Kim YK, Yoo WI, Lee SH and Lee MY (2005) Proteomic analysis of cadmium-induced protein profile alterations from marine alga *Nannochloropsis oculata*. Ecotoxicology, **14**, 589 – 596.

∴ Kirkham MB (2006) Cadmium in plants on polluted soils: Effects of soil factors, hyperaccumulation and amendments. *Geoderma*, **137**, 19 – 32.

∴ Klang-Westin E and Erikson J (2003) Potential of Salix as phytoextractor for Cd on moderately contaminated soils. Plant Soil, **249**, 127 – 137.

∴ Knief C, Delmotte N and Vorholt JA (2011) Bacterial adaptation to live in association with plants – a proteomic perspective from culture to *in situ* conditions. Proteomics, **11**, 15: 3086 – 3105.

∴ Ko K and Cashmore AR (1989) Targeting of proteins to the thylakoid lumen by the bipartite transit peptide of the 33 kd oxygen evolving protein. *EMBO Journal*, **8**, 3187 – 3194.

∴ Kosová K, Vítímvás P, Prásil IT and Renaut J (2011) Plant proteome changes under abiotic stress – contribution of proteomics studies to understanding plant stress response, Journal of Proteomics, **74**, 1301 – 1322.

∴ Krämer U, Talke IN and Hanikenne M (2007) Transition metal transport. FEBS Letters, **581**, 2263 – 2272.

∴ Krznaric E, Verbruggen N, Wevers JHL, Carleer R, Vangronsveld J and Colpaert JV (2009) Cd-tolerant *Suillus luteus*: A fungal insurance for pines exposed to Cd. Environmental pollution, **157**, 1581 – 1588.

∴ Krznaric E, Wevers JHL, Cloquet C, Vangronsveld J, Vanhaecke F and Colpaert JV (2010) Zn pollution counteracts Cd toxicity in metal-tolerant ectomycorrhizal fungi and their host plant, *Pinus sylvestris*. Environmental Biology, **12**, 2133 – 2141.

∴ Kumar V, Parkhi V, Kenerley CM and Rathore KS (2009) Defense-related gene expression and enzyme activities in transgenic cotton plants expressing an endochitinase gene from Trichoderma virens in response to interaction with Rhizoctonia solani. *Planta*, **230**, 277 – 291.

∴ Kvint K, Nachin L, Diez A and Nyström T (2003) The bacterial universal stress protein: function and regulation. *Current Opinion in Microbiology*, **6**, 140 – 145.

∴ Lagriffoul A, Mocquot B, Mench M and Vangronsveld J (1998) Cadmium toxicity effects on growth, mineral and chlorophyll contents and activities of stress-related enzymes in young maize plants (*Zea mays* L.) Plant Physiology and Biochemistry, **200**, 241 – 250.

∴ Lambertson D, Chen L and Madura K (1999) Pleiotropic growth and proteolytic defects caused by the loss of the proteasome-interacting factors rad23 and rpn10 of *Saccharomyces cerevisae*. *Genetics*, **153**, 69 – 79.

∴ Lane TW and Morel FM (2000) A biological function for Cd in marine diatomeas. *Proceedings* of the National Academy of Science of the USA, **97**, 4627 – 4631.

∴ Langella F, Stark R, Weist A, Grawunder A and Kothe E (2012) Microbial consortia enhancing plant growth for the remediation of heavy metal contaminated sites. 9th International Phytotechnology Society Conference, Book of Abstracts, p9.

∴ Lanquar V, Kuhn L, Lelièvre F, Khafif M, Espagne C, Bruley C, Barbier-Brygoo H, Garin J and Thomine S (2007) ^{15}N-metabolic labelling for comparative plasma membrange proteomics in *Arabidopsis* cells. Proteomics, **7**, 750 – 754.

∴ Lasat MM (2002) Phytoextraction of toxic metals: a review of biological mechanisms. Journal of Environmental Quality, **31**, 109 – 120.

∴ Laureysens I, Blust R, De Temmerman L, Lemmens C and Ceulemans R (2004) Clonal variation in heavy metal accumulation and biomass production in a poplar coppice culture: I. Seasonal variation in leaf, wood and bark concentrations. Environmental Pollution, **131**, 485 – 494.

∴ Laureysens I, De Temmerman L, Hastir T, Van Gysel M and Ceulemans R (2005) Clonal variation in heavy metal accumulation and biomass production in a poplar coppice culture. II. Vertical distribution and phytoextraction potential. Environmental Pollution, **133**, 541 – 551.

∴ Lazarus M (2010) Cd and selenium interaction in mammals. Archives of Inducstrial Hygiene and Toxicology, **61**, 3: 357 – 369.

∴ Lebeau T, Braud A and Jézéquel K (2008) Performance of bioaugmentation-assisted phytoextraction applied to metal contaminated soils: A review. Environmental Pollution, **153**, 497 – 522.

∴ Lee K, Bae DW, Kim SH, Han HJ, Liu X, Park HC, Lim CO, Lee SY and Chung WS (2010) Comparative proteomic analysis of the short-term responses of rice roots and leaves to cadmium. Journal of Plant Physiology, **167**, 161 – 168.

∴ Legatzki A, Grass G, Anton A, Rensing C and Nies DH (2003) Interplay of the Czc System and two P-type ATPases in conferring metal resistance to *Ralstonia metallidurans*. Journal of Bacteriology, **185**, 15: 4354 – 4361.

∴ Lemos MFL, Soares AMVM, Correia AC, Esteves AC (2010) Proteins in ecotoxicology – How, why and why not? Proteomics, **10**, 873 – 887.

∴ Leshem YY and Kuiper PJC (1996) Is there a GAS (general adaptation syndrome) response to various types of environmental stress? Biologia Plantarum, **38**, 1 – 18.

∴ Liang HM, Lin TH, Chiou JM and Yeh KC (2009) Model evaluation of the phytoextraction potential of heavy metal hyperaccumulators and non-hyperaccumulators. Environmental Pollution, **157**, 1945 – 1952.

∴ Lichtenthaler HK (1996) Vegetation Stress: an introduction to the stress concept in plants. *Journal of Plant Physiology*, **148**, 4 – 14.

∴ Lilley KS, Deery MJ and Gatto L (2011) Challenges for proteomics core facilities. Proteomics, **11**, 1017 – 1025.

∴ Link AJ, Eng J, Schieltz DM, Carmack E, Mize GJ, Morris DR, Garvik BM and Yates JR (1999) Direct analysis of protein complexes using mass spectrometry. Nature Biotechnology, **17,** 676 – 682.

∴ Liu X, Zhang S, Shan X and Zhu YG (2005) Toxicity of arsenate and arsenite on germination seedling growth and amylolytic activity of wheat. Chemosphere, **61**, 293 – 301.

∴ Lodewyckx C, Mergeay M, Vangronsveld J, Clijsters H and van der Lelie D (2002a) Isolation, characterisation, and identification of bacteria associated to the zinc hyperaccumulator Thlaspi caerulescens subsp. Calaminaria. International Journal of Phytoremediation, **4**, 2: 101 – 115.

∴ Lodewyckx C, Vangronsveld J, Porteous F, Moore ERB, Taghavi S, Mezgeay M and van der Lelie D (2002b) Endophytic bacteria and their potential applications. Critical Reviews in Plant Sciences, **21**, 583 – 606.

∴ Lombi E, Zhoa FJ, Dunham SJ and McGrath SP (2000) Cadmium accumulation in population of Thlaspi caerulescens and Thlaspi goesingense. New Phytology, **145**, 11 – 20.

∴ Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with the folin phenol reagent. Journal of Biological Chemistry, **193**, 265 – 275.

∴ Lucy M, Reed E and Blick BR (2004) Applications of free living plant growth-promoting rhizobacteria. Antonie van Leeuwenhoek, **86**, 1 – 25.

∴ Luparello C, Sirchia R and Longo A (2011) Cd as a transcriptional modulator in human cells. Critical Reviews in Toxicology, **41**, 1: 75 – 82.

∴ Lytle BL, Song J, de la Cruz NB, Peterson FC, Johnson KA, Bingman CA, Philips GN Jr and Volkman BF (2009) Structures of two Arabidopsis thaliana major latex preoteins represent novel helix-grip folds. Proteins, **76**, 237 – 243.

∴ Macnair MR, Tilstone GH and Smith SE (2000) The genetics of metal tolerance and accumulation in higher plants. In: Terry N, Banuelos G (eds.) Phytoremediation of contaminated soil and water. CRC Press LLC, Boca Raton, Florida, USA, pp. 235 – 250.

∴ MacRobbie EA and Kurup S (2007) Signalling mechanisms in the regulation of vacuolar ion release in guard cells. New Phytologist, **175**, 630 – 640.

∴ Madmony A, Chernin L, Pleban S, Peleg E and Riov J (2005) Enterobacter cloacae, an abligatory endophyte of pollen grains of Mediterranean pines. Folia Microbiologica, **50**, 209 – 216.

∴ Martinoia E, Maeshima M and Neuhaus HE (2007) Vacuolar transporters and their essential role in plant metabolism. Journal of Experimental Botany, **58**, 1: 83 – 102.

∴ Masood A, Iqbal N and Khan NA (2012) Role of ethylene in alleviation of Cd-induced photosynthetic capacity inhibition by sulphur in mustard. Plant, Cell and Environment, **35**, 524 – 533.

∴ Mastretta C, Barac T, Vangronsveld J, Newman L, Taghavi S and van der Lelie D (2006) Endophytic bacteria and their potentional application to improve the phytoremediation of contaminated environments. Biotechnology and Genetic Engeneering Reviews, **23**, 175 – 207.

∴ Mastretta C, Taghavi S, van der Lelie D, Mengoni A, Galardi F, Barac T, Boulet J, Weyens N and Vangronsveld J (2009) Endophytic bacteria from seeds of nicotiana tabacum can reduce cadmium phytotoxicity. International Journal of Phytoremediation, **11**, 251 – 267.

∴ Mathesius U (2009) Comparative proteomic studies of root-microbe interactions. Journal of Proteomics, **72**, 353 – 366.

∴ Matovic V, Buha A, Bulat Z and Dukic-Cosic D (2011) Cd toxicity revisited: focus on oxidative stress induction and interactions with zinc and magnesium. Archives Of Industrial Hygiene and Toxicology, **62**, 65 – 76.

∴ Mauch F and Staehelin LA (1989) Functional implications of the subcellular localization of ethylene induced chitinase and beta-1,3-glucanase in bean leaves. *The Plant Cell*, **1**, 447 – 457.

∴ Mc Cashin BG (2000) Induction of nitrate reductase in plant shoots. In: Karcher SJ (ed.) Tested studies for laboratory teaching. Proceedings of the 21st Workshop/Conference of the Association for Biology Laboratory Education (ABLE), pp. 193 – 224.

∴ McLaughlin MJ, Tiller KG, Naidu R and Stevens DP (1996) Reviewed: The behavior and impact of contaminants in fertilizers. Australian Journal of Soil Research, **34**, 1 – 54.

∴ Meers E, Tack FMG, Van Slucken S, Ruttens A, Vangronsveld J and Verloo MG (2008) Chemically assisted phytoextraction: A review of potential soil amendments for increasing plant uptake of heavy metals. International Journal of Phytoremediation, **10**, 390 – 414.

∴ Meers E, Van Slycken S, Adriaensen K, Ruttens A, Vangronsveld J, Du Laing G, Witters N, Thewys T and Tack FMG (2010) The use of bio-energy crops (*Zea mays*) for ‘phytoattenuation’ of heavy metals on moderately contaminated soils: A field experiment. Chemosphere, **78**, 35 – 41.

∴ Memisi N, Zujovic M, Bogdanovic V, Tomic Z and Petrovic MP (2008) The influence of presence of Cd and arsenic in feedmeal on production and reproduction traits of goats. Biotechnology in Animal Husbandry, **24**, 39 – 47.

∴ Mench M, Vangronsveld J, Didier V and Clijsters H (1994) Evaluation of metal mobility, plant availability and immobilization by chemical agents in a limed silty soil. Environmental Pollution, **86**, 279 – 286.

∴ Mench M, Schwitzgue´bel JP, Schroeder P, Bert V, Gawronski S and Gupta S (2009) Assessment of successful experiments and limitations of phytotechnologies: contaminant uptake, detoxification and sequestration and consequences for food safety. *Environmental Science and Pollution Research*, **16**, 876 – 900.

∴ Meplan C, Mann K and Hainaut P (1999) Cd induces conformational modifications of wild-type p53 and suppresses p53 response to DNA damage in cultured cells. Journal of Biological Chemistry, **274,** 31663 – 31670.

∴ Mertens J, Vervaeke P, Meers E and Tack (2006) Seasonal changes of metals in willow (Salix sp.) stands for phytoremediation on dredged sediment. *Environmental Science and Technology*, **40**, 1962 – 1968.

∴ Meurer J, Berger A and Westhoff P (1996) A nuclear mutant of Arabidopsis with impaired stability on distinct transcripts of the plastig psbB, psbD/C, ndhH and ndhC operons. *Plant Cell*, **8**, 1193 – 1207.

∴ Meurer J, Lezhneva L, Amann K, Gödel M, Bezhani S, Sherameti I and Oelmüller R (2002) A peptide chain release factor 2 affects the stability of UGA-containing transcripts in Arabidopsis chloroplasts. *Plant Cell*, **14**, 3255 – 3269.

∴ Miller I, Eberini I and Gianazza E (2010) Other than IPG-DALT: 2-DE variants. Proteomics, **10**, 586 – 610.

∴ Mills RF, Francini A, da Rocha PSCF, Baccarini PJ, Aylett M,Krijger GC and Williams LE (2005) The plant P-1B-type ATPase AtHMA4transports Zn and Cd and plays a role in detoxification of transition metals supplied at elevated levels. FEBS Letters, **579,** 783 – 791.

∴ Miransari M (2010) Contribution of arbuscular mycorrhizal symbiosis to plant growth under different types of soil stress. Plant Biology, **12**, 563 – 569.

∴ Mittler R, Venderauwera S, Gollery M and Van Breusegem F (2004) Reactive gene network of plants. Trends in Plant Science, **9**, 490 – 498.

∴ Moulis JM (2010) Cellular mechanisms of Cd toxicity related to the homeostasis of essential metals. BioMetals, **23**, 877 – 896.

∴ Nachin L, Nannmark U and Nyström T (2005) Differential roles of the universal stress proteins of *Escherichia coli* in oxidative stress resistance, adhesion and motility. *Journal of Bacteriology*, **187**, 6265 – 6272.

∴ Nachtegaal M, Marcus MA, Sonke JE, Vangronsveld J, Livi KJT, van der Lelie N and Sparks DL (2005) Effects of *in situ* remediation on the speciation an bioavailability of zinc in a smelter contaminated soil. Geochimica and Cosmochimica Acta, **69**, 19: 4649 – 4664.

∴ Nagano T, Kojima K, Hisabori T, Hayashi H, Morita EH, Kanamori T, Miyagi T, Ueda T and Nishiyama Y (2012) Elongation factor G is a critical target during oxidative damage to the translation system of Escherichia coli. *Journal of Biological Chemistry*, **287**, 28697 – 28704.

∴ Nawrot TS, Plusquin M, Hogervorst J, Roels HA, Celis H, Thijs L, Vangronsveld J, Van Hecke E and Staessen JA (2006) Environmental exposure to cadmium and risk of cancer: a prospective population-based study. *The Lancet Oncology*, **7**, 119 – 126.

∴ Nawrot TS, Van Hecke E, Thijs L, Richart T, Kuznetsova T, Jin Y, Vangronsveld J, Roels HA and Staessens JA (2007) Cadmium-related mortality and long-term secular trends in the cadmium body burden of an environmentally exposed population. *Environmental Health Perspectives*, **116**, 1620 – 1628.

∴ Nawrot TS, Staessen JA, Roels HA, Munters E, Cuypers A, Richart T, Ruttens A, Smeets K, Clijsters H and Vangronsveld J (2010) Cd exposure in the population: from health risks to strategies of prevention. BioMetals, **23**, 769 – 782.

∴ Nesvizhskii AI, Keller A, Kolker E and Aebersold R (2003) A statistical model for identifying proteins by tandem mass spectrometry. Analytical Chemistry, **75**, 4646 – 4658.

∴ Neter J, Kutner MH, Nachtsheim CJ and Wasserman W (1996) Simple Linear regression. Fouth edition. Irwin, USA, pp. 95-152.

∴ Nies DH, Nies A, Chu L and Silver S (1989) Expression and nucleotide sequence of a plasmid determined divalent cation efflux system from *Alcaligenes eutrophus*. Proceedings of the National Academy of Science of the USA, **86**, 7351 – 7355.

∴ Nies DH (1992) CzcR and CzcD, gene products affecting regulation of resistance to cobalt, zinc and Cd (czc system) in *Alcaligenes eutrophus*. Journal of Bacteriology, **174**, 8102 – 8110.

∴ Noctor G and Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. Annual Review on Plant Physiology and Plant Molecular Biology, **49**, 249 – 279.

∴ Nriagu J and Pacyna J (1988) Quantitative assessment of worldwide contamination of air, water and soils by trace metals, Nature, **333**, 134 – 139.

∴ Ohta H and Cherian MG (1991) Gastrointestinal absorption of Cd and metallothionein. Toxicology and Applied Pharmacology, **107**, 63 – 72.

∴ Ortolan TG, Tongaonkar P, Lambertson D, Chen L, Schauber C and Madura K (2000) The DNA repair protein rad23 is a negative regulator of multi-ubiquitin chain assembly. *Nature Cell Biology*, **2**, 601 – 608.

∴ Perfus-Barbeoch L, Leonhardt N, Vavaddeur A and Forestier C (2002) Heavy metal toxicity: cadmium permeates through calcium channels and disturbs the plant water status. Plant Journal, **32**, 539 – 548.

∴ Petrak J, Ivanek R, Toman O, Cmejla R, Cmejlova J, Vyoral D, Zivny J and Vulpe CD (2008) Déjà vu in proteomics. A hit parade of repeatedly identified differentially expressed proteins. Proteomics, **8**, 1744 – 1749.

∴ Pietrini F, Zacchini M, Iori V, Pietrosanti L, Bianconi D and Massacci A (2010) Screening of poplar clones for Cd phytoremediation using photosynthesis, biomass and Cd content analyses. International Journal of Phytoremediation, **12**, 105 – 120.

∴ Pinchas F, Paresh R and Beriozkin A (2012) Ligand enhanced heavy metal uptake by fast growing trees under controlled deficit irrigation - Results of batch and lysimeter studies. *9th International Phytotechnology Society Conference*, Book of Abstracts, p83.

∴ Pirttilä AM, Laukkanen H, Pospiech H, Myllylä R and Hohtola A (2000) Detection of intracellular bacteria in the buds of Scots pine (Pinus sylvestris L.) by in sity hybridization. Applied and environmental Microbiology, **66**, 3073 – 3077.

∴ Plomion C, Lalanne C, Claverol S, Meddour H, Kohler A, Bogeat-Triboulot MB, Barre A, Le Provost G, Dumazet H, Jacob D, Bastien C, Dreyer E, de Daruvar A, Guehl JM, Schmitter JM, Martin F and Bonneu M (2006) Mapping the proteome of poplar and application to the discovery of drought-stress responsive proteins. Proteomics, **6**, 6509 – 6527.

∴ Prasad MNV (1995) Cadmium toxicity and tolerance in vascular plants. Environmental and Experimental Botany, **35**, 525 – 545.

∴ Prather CW and Sisler EC (1972) Glycine and glyoxylate decarboxylation in *Nicotiana rustica*. *Phytochemistry*, **11**, 1637 – 1647.

∴ Price DJ and Joshi JG (1983) Ferritin. Binding of beryllium and other divalent metal ions. Journal of Biological Chemistry, **258**, 10873 – 10880.

∴ Pulford ID and Dickinson NM (2006) Phytoremediation technologies using trees. In: Prasad MNV, Sajwan KS and Naïdu R (eds.) Trace elements in the environment: biogeochemistry, biotechnology and bioremediation. CRC Press LLC, Boca Raton, Florida, USA, pp. 375 – 395.

∴ Rauser WE (1995) Phytocheltains and related peptides. Structure, biosynthesis and function. Plant Physiology, **109**, 1141 – 1149.

∴ Rauser WE (2003) Phytochelatin-based complexes bind various amounts of Cd in maize seedlings depending on the time of exposure, the concentration of Cd and the tissue, New Phytologist, **158**, 269 – 278.

∴ Reeves RD and Baker AJM (1999) Metal-accumulating plants. In: Raskin I and Ensley BD (eds.) Phytoremediation of Toxic Metals: Using Plants to Clean up the Environment. John Wiley & Sons Inc., New York, USA, pp. 193 – 229.

∴ Reisinger F and Martens L (2009) Database on Demand – An online tool for the custom generation of FASTA-formatted sequence databases. Proteomics, **9**, 4421 – 4424.

∴ Remans T, Thijs S, Truyens S, Weyens N, Schellingen K, Keunen E, Gielen H, Cuypers A and Vangronsveld J (2012) Understanding the development of roots exposed to contaminants and potential of plant-associated bacteria for optimization of growth. Annals of Botany, **110**, 239 – 252.

∴ Renard BY, Kirchner M, Monigatti F, Ivanov AR, Rappsilber J, Winter D, Steen JAJ, Hamprecht FA and Steen H (2009) When less can yiels more – Computational preprocessing of MS/MS spectra for peptide identification. Proteomics, **9**, 4978 – 4984.

∴ Repetto O, Bestel-Corre G, Dumas-Gaudot E, Berta G, Gianinazzi-Pearson V and Gianinazzi S (2003) Targeted proteomics to identify Cd-induced protein modifications in Glomus mosseae-inoculated pea roots. New Phytologist, **157**, 555 – 567.

∴ Riesen O and Feller U (2005) Redistribution of nickel, cobalt, manganese, zinc and Cd via the phloem in young and maturing wheat. Journal of Plant Nutrition, **28,** 421 – 30.

∴ Robinson BH, Mills TM, Petit D, Fung LE, Green SR and Clothier BE (2000) Natural and induced Cd-accumulation in poplar and willow: Implications for phytoremediation. Plant and Soil, **227**, 301 – 306.

∴ Rodríguez-Celma J, Rellán-Álvarez R, Abadía A, Abadía J and López-Millán (2010) Changes induded by two levels of cadmium toxicity in the 2-DE protein profile of tomato roots. Journal of Proteomics, **73**, 1694 – 1706.

∴ Rodríguez-Serrano M, Romero-Puertas MC, Zabalza A, Corpas FJ, Gómez M, del Río LA and Sandalio LM (2006) Cadmium effect on oxidative metabolism of pea (Pisum sativum L.) roots. Imaging of reactive oxygen species and nitric oxid accumulation in vivo. *Plant, Cell & Environment*, **29**, 1532 – 1544.

∴ Rodríguez-Serrano M, Romero-Puertas MC, Pazmino DM, Testillano PS, Risueno MC, del Río LA and Sandalio LM (2009) Cellular response of pea plants to cadmium toxicity: cross talk between reactive oxygen species, nitric oxide and calcium. *Plant Physiology*, **150**, 229 – 243.

∴ Romano PGN, Horton P and Gray JE (2004) The Arabidopsis cyclophilin gene family. *Plant Physiology*, **134**, 1268 – 1282.

∴ Root RA, Miller RJ and Koeppe DE (1975) Uptake of Cd—its toxicity and effect on the iron-to-zinc ratio in hydroponically grown corn. Journal of Environmental Quality, **4**, 473 – 476.

∴ Rosenbleuth M and Martinez-Romero E (2006) Bacterial endophytes and their interactions with hosts. The American Phytopathological Society, **19**, 827 – 837.

∴ Rossignol M, Peltier J-B, Mock H-P, Matros A, Maldonado AM and Jorrín JV (2006) Plant proteome analysis: a 2004 – 2006 update. Proteomics, **20**, 5529 – 5548.

∴ Roth U, von Roepenack-Lahaye E and Clemens S (2006) Proteome changes in *Arabidopsis thaliana* roots upon exposure to Cd^{2+}. Journal of Experimental Botany, **57**, 4003 – 4013.

∴ Ruttens A, Mench M, Colpaert JV, Boisson J, Carleer R and Vangronsveld (2006) Phytostabilization of a metal contaminated sandy soil. I: Influence of compost and/or inorganic metal immobilizing soil amendments on phytotoxicity and plant availability of metals. Environmental Pollution, **144**, 524 – 532.

∴ Ruttens A, Adriaensen K, Meers E, De Vocht A, Geebelen W, Carleer R and Mench M (2010) Long-term sustainability of metal immobilization by soil

amendments: cyclonic ashed versus lime addition. Environmental Pollution, **158**, 1428 – 1434.

∴ Ruttens A, Boulet J, Weyens N, Smeets K, Adriaensen K, Meers E, Van Slycken S, Tack F, Meiresonne L, Thewys T, Witters N, Carleer R, Dupae J and Vangronsveld J (2011) Short rotation coppice culture of willow and poplars as energy crops on metal contaminated agricultural soils. International Journal of Phytoremediation, **13**, 194 – 207.

∴ Ryan RP, Germaine K, Franks A, Ryan DJ and Dowling DN (2008) Bacterial endophytes: recent developments and applications. FEMS Microbiology Letters, **278**, 1 – 9.

∴ Salt DE, Prince RC, Pickering IJ and Raskin I (1995) Mechanisms of Cd mobility and accumulation in Indian mustard, Plant Physiology, **109**, 1427 – 1433.

∴ Salisbury FB and Ross CW (1992) Assimilation of Nitrogen and sulphur. In: Salisbury FB and Ross CW (eds) Plant physiology. Wadsworth Publishing Company, Belmont, CA, USA, pp. 289 – 307.

∴ Salmi J, Nyman TA, Nevalainen OS and Aittokallio T (2009) Filtering strategies for improving protein identification in high-throughput MS/MS studies. Proteomics, **9**, 848 – 860.

∴ Salt DE, Smith RD and Raskin I (1998) Phytoremediation. Annual Review of Plant Physiology and Plant Molecular Biology, **49**, 643 – 668.

∴ Sanità di Toppi L and Gabbrielli R (1999) Response to cadmium in higher plants. Environmental and Experimental Botany, **41**, 105 – 130.

∴ Saravanan RS and Rose JKC (2004) A critical evaluation of sample extraction techniques for enhanced proteomis analysis of recalcitrant plant tissues**.** Proteomics, **4**, 2522 – 2532.

∴ Sarkar NK, Kundnani P and Grover A (2012) Functional analysis of Hsp70 superfamily proteins of rice (Oryza sativa). *Cell Stress and Chaperones*, [Epub ahead of print], DOI: 10.1007/s12192-012-0395-6

∴ Sarma AD, Oehrle NM and Emerich DW (2008) Plant protein isolation and stabilization for enhanced resolution of two-dimensional polyacrylamide gel electrophoresis. Analytical Biochemistry, **379**, 192 – 195.

∴ Sarry JE, Kuhn L, Ducriux C, Lafaye A, Junot C, Hugouvieux V, Jourdain A, Bastien O, Fievet JB, Vailhen D, Amekraz B, Moulin C, Ezan E, Garin J and Bourguignon J (2006 The early responses of Arabidopsis thaliana cells to cadmium exposure explored by protein and metabolite profiling analysis. Proteomics, **6**, 2180 – 2198.

∴ Sarwar N, Saifullah, Malhi SS, Zia MH, Naeem A, Bibi S and Farid G (2010) Role of mineral nutrition in minimizing Cd accumulation by plants. Journal of the Science of Food and Agriculture, **90**, 925 – 937.

∴ Sauve S, Norvell WA, McBride M and Hendershot W (2000) Speciation and complexation of Cd in extracted soil solutions, Environmental Science and Technology, **34**, 291 – 296.

∴ Schneider T, Schellenberg M, Meyer S, Keller F, Gehrig P, Riedel K, Lee Y and Martinoia E (2009) Quantitative detection of changes in the leaf-mesophyll tonoplast proteome in dependency of a cadmium exposure of barley (*Hordeum vulgare* L.) plants. Proteomics, **9**, 2668 – 2677.

∴ Schützendübel A and Polle A (2002a) Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. Journal of Experimental Botany, **53**, 1351 – 1365.

∴ Schützendübel A, Nikolova P, Rudolf C and Polle A (2002b) Cd and H_2O_2-induced oxidative stress in *Populus x canescens* roots. Plant Physiology and Biochemistry, **40**, 577 – 584.

∴ Schwyn B and Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. Analytical Biochemistry, **160**, 47 – 56.

∴ Searle BC, Turner M and Nesvizhskii AI (2008) Improving sensitivity by probabilistically combining results from multiple MS/MS search methodologies. Journal of Proteome Research, **7**, 245 – 253.

∴ Searle BC (2010) Scaffold: A bioinformatic tool for validating MS/MS-based proteomic studies. Proteomics, **10**, 1265 – 1269.

∴ Sela-Buurlage MB, Ponstein AS, Bres-Vloemans SA, Melchers LS, van den Elzen PJM and Cornelissen BJC (1993) Only specific tobacco (Nicotiana tabacum) chitinases and beta-1,3-glucanases exhibit antifungal activity. *Plant Physiology*, **101**, 857 – 863.

∴ Selosse MA, Baudoin E and Vandenkoornhuyse P (2004) Symbiotic microorganisms, a key for ecological success and protection of plants. Comptes Rendus Biologies, **327**, 639 – 648.

∴ Semane B, Cuypers A, Smeets K, Van Belleghem F, Horemans N, Schat H and Vangronsveld J (2007) Cadmium responses in *Arabidopsis thaliana*: glutathione metabolism and antioxidative defence system. Physiologia Plantarum, **129**, 519 – 528.

∴ Semane B, Dupae J, Cuypers A, Noben JP, Tuomainen M, Tervahauta A, Kärenlampi S, Van Belleghem F, Smeets K and Vangronsveld J (2010) Leaf proteome responses of *Arabidopsis thaliana* exposed to mild cadmium stress. Journal of Plant Physiology, **167**, 247 – 254.

∴ Seth CS, Remans T, Keunen E, Jozefczak M, Gielen H, Opdenakker K, Weyens N, Vangronsveld J and Cuypers A (2012) Phytoextraction of toxic metals: a central role for glutathione. Plant, Cell and Environment, **35**, 334 – 346.

∴ Shah K and Dubey RS (1995) Effect of Cd on RNA level as well as activity and molecular forms of ribonuclease in growing rice seedlings. Plant Physiology and Biochemistry, **33**, 577 – 584.

∴ Sharma M, Gautam KH and Handique AK (2006) Toxic heavy metal stress in paddy: metal accumulation profile and development of novel stress protein in seed. Indian Journal of Plant Physiology, **11**, 227 – 233.

∴ Sharma SS and Dietz KJ (2008) The relationship between metal toxicity and cellular redox imbalance. Trends in Plant Science, **14,** 43 – 50.

∴ Shenker M, Fan TWM and Crowley DE (2001) Phytosiderophores influence on Cd mobilization and uptake by wheat and barley plants. Journal of Environmental Quality, **30**, 2091 – 2098.

∴ Shevchenko A, Wilm M, Vorm O and Mann M (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Analytical Chemistry, **68**, 850 – 858.

∴ Shigeoka S, Ishikawa T, Tamoi M, Miyagawa Y, Takeda T, Yabuta Y and Yoshimura K (2002) Regulation and function of ascorbate peroxidise isoenzymes. *Journal of Experimental Botany*, **53**, 1305 – 1319.

∴ Siegel S and Castellan NJ (1988) Non parametric statistics for behavioral sciences. McGraw-Hill Book Compagny, Singapore, India, ISBN: 0070573573.

∴ Simmler M, Ciadamidaro L, Dickinson N, Madejon P, Schulin R and Robinson B (2012) Managing the phytoaccumulation of cadmium using lignite. 9th International Phytotechnology Society Conference, Book of Abstracts, p139.

∴ Singer AC, van der Gast CJ and Thompson IP (2005) Perspetives and vision for strain selection in bioaugmentation. TRENDS in Biotechnology, **23**, 2: 74 – 77.

∴ Smeets K, Cuypers A, Lambrechts A, Semane B, Hoet P, Van Laere A and Vangronsveld J (2005) Induction of oxidative stress and antioxidative mechanisms in *Phaseolus vulgaris* after Cd application. Plant Physiology and Biochemistry, **43**, 437 – 444.

∴ Smeets K, Ruytinx J, Semane B, Van Belleghem F, Remans T, Van Sanden S, Vangronsveld J and Cuypers A (2008a) Cadmium-induced transcriptional and metabolic alterations related to oxidative stress. Environmental and Experimental Botany, **63**, 1 – 8.

∴ Smeets K, Ruytinx J, Van Belleghem F, Semane, B, Lin D, Vangronsveld J and Cuypers A (2008b) Critical evaluation and statistical validation of a hydroponic culture system for Arabidopsis thaliana. Plant Physiology and Biochemistry, **46**, 212 – 218.

∴ Smeets K, Opdenakker K, Remans T, Van Sanden S, Van Belleghem F, Semane B, Horemans N, Guisez Y, Vangronsveld J and Cuypers A (2009) Oxidative stress-related responses at transcriptional and enzymatic level after exposure to Cd or Cu and in a multi-pollution context. Journal of Plant Physiology, **166**, 149 – 177.

∴ Smolders E and McLaughlin MJ (1996) Effect of Cl on Cd uptake by Swiss chard in nutrient solutions. Plant and Soil, **179**, 57 – 64.

∴ Sobkowiak R and Deckert J (2003) Cd-induced changes in growth and cell cycle gene expression in suspension culture cells of soybean. Plant Physiology and Biochemistry, **41**, 767 – 772.

∴ Sobkowiak R and Deckert J (2004) The effect of Cd on cell cycle control in suspension culture cells of soybean. Acta Physiologiae Plantarum, **26**, 335 – 344.

∴ Sobkowiak R and Deckert J (2006) Proteins induced by cadmium in soybean cells. Journal of Plant Physiology, **163**, 1203 – 1206.

∴ Srivastava S, Verma PC, Chaundhry V, Singh N, Abhilash PC, Kumar KV, Sharma N and Singh N (2012) Influence of inoculation of arsenic-resistant Staphylococcus arlettae on growth and arsenic uptake in Brassica juncea (L.) Czern. Var. R-46. *Journal of Hazardous Materials*, **17**, http://dx.doi.org/10.1016/j.jhazmat.2012.08.019

∴ Stals M, Thijssen E, Vangronsveld J, Carleer R, Schreurs S and Yperman J (2010) Flash pyrolyse of heavy metal contaminated biomass from phytoremediation: influence of temperature, entrained flow and wood/leaves blended pyrolysis on the behaviour of heavy metals. Journal of Analytical and Applied Pyrolysis, **87**, 1 – 7.

∴ Stroinski A (1997) Effects of Cd on the host-pathogen system. V. Effect of exogenous dicyclohexylamine on potato tubers (*Solanum tuberosum* L.), Cd and *Phytophtora infestans* relations. Journal of Plant Physiology, **150**, 178 – 183.

∴ Sun W, Li F, Wang J, Zheng D and Gao Y (2004) AMASS: software for automatically validating the quality of MS/MS spectrum from SEQUEST results. Molecular and Cellular Proteomics, **12**, 1194 - 1199.

∴ Taghavi S, Mergeay M, Nies D and van der Lelie D (1997) *Alcaligenes eutrophus* as a model system for bacterial interactions with heavy metals in the environment. Research in Microbiology, **148**, 6: 536 – 551.

∴ Taghavi S, Barac T, Greenberg B, Borremans B, Vangronsveld J and van der Lelie D (2005) Horizontal gene transfer to endogenous endophytic bacteria from poplar improved phyto-remediation of toluene. Applied Environmental Microbiology, **71,** 8500 – 8505.

∴ Taghavi S, Garafola C, Monchy S, Newman L, Hoffman A, Weyens N, Barac T, Vangronsveld J and van der Lelie D (2009) Genome survey and characterization of endophytic bacteria exhibiting a beneficial effect on growth and development of poplar trees. Applied and Environmental Microbiology, **75**, 3: 748 – 757.

∴ Taiz L and Zeiger E (2002) Plant Physiology. Sinauer Associated Inc., Sunderland, Massachusetts, pp.690. ISBN 0-87893-823-0

∴ Taylor CF, Paton NW, Lilley KS, Binz PA, Julian RK Jr, Jones AR, Zhu W, Apweiler R, Aebersold R, Deutsch EW, Dunn MJ, Heck AJ, Leitner A, Macht M, Mann M, Martens L, Neubert TA, Patterson SD, Ping P, Seymour SL, Souda P, Tsugita A, Vandekerckhove J, Vondriska TM, Whitelegge JP, Wilkins MR, Xenarios I, Yates JR 3rd and Hermjakob H (2007) The minimum information about a proteomics experiment (MIAPE). Nature Biotechnology, **25**, 887 - 993.

∴ Taylor CF, Binz PA, Aebersold R, Affolter M, Barkovich R, Deutsch EW, Horn DM, Hühmer A, Kussmann M, Lilley K, Macht M, Mann M, Müller D, Neubert TA, Nickson J, Patterson SD, Raso R, Resing K, Seymour SL, Tsugita A, Xenarios I, Zeng R and Julian RK Jr (2008) Guidelines for reporting the use of mass spectrometry in proteomics. Nature Biotechnology, **26**, 860 - 861.

∴ Tharakan R, Edwards N and Graham DRM (2010) Data maximization by multipass analysis of protein mass spectra. Proteomics, **10**, 1160 – 1171.

∴ Thévenod F (2010) Catch me if you can! Novel aspects of Cd transport in mammalian cells. Biometals, **23**, 5: 857 – 875.

∴ Thiellement H, Zivy M and Darmeval C (2006) Plant Proteomics: Methods and Protocols. Humana Press Inc., New Jersey, pp. 9 – 14.

∴ Thomas H and Shevchenko A (2008) Simplified validation of borderline hits of database searches. Proteomics, **8**, 4173 – 4177.

∴ di Toppi LS and Gabbrielli R (1999) Response to Cd in higher plants. Environmental and Experimental Botany, **41**, 105 – 130.

∴ Truyens S, Weyens N, Cuypers A and Vangronsveld J (2012) Changes is the population of seed bacteria of transgenerationally Cd-exposed Arabidopsis thaliana. *Plant Biology Stuttgart*, [Epub ahead of print], doi: 10.1111/j.1438-8677.2012.00711.x., ISSN 1435 – 8603.

∴ Tuomainen MH, Nunan N, Lehesranta SJ, Tervahaua AI, Hassinen VH, Schat H, Koistinen KM, Auriola S, McNicol J and Kärenlampi SO (2006)

Multivariate analysis of protein profiles of metal hyperaccumulator Thlaspi caerulescens accessions. Proteomics, **6**, 3696 – 3706.

∴ Tuskan GA, DiFazio S, Jansson S, Bohlmann J, Grigoriev I, Hellsten U, Putnam N, Ralph S, Rombauts S, Salamov A, Schein J, Sterck L, Aerts A, Bhalerao RR, Bhalerao RP, Blaudez D, Boerjan W, Brun A, Brunner A, Busov V, Campbell M, Carlson J, Chalot M, Chapman J, Chen GL, Cooper D, Coutinho PM, Couturier J, Covert S, Cronk Q, Cunningham R, Davis R, Degroeve S, Déjardin A, dePamphilis C, Detter J, Dirks B, Dubchak I, Duplessis S, Ehlting J, Ellis B, Gendler K, Goodstein D, Gribskov M, Grimwood J, Groover A, Gunter L, Hamberger B, Heinze B, Helariutta Y, Henrissat B, Holligan D, Holt R, Huang W, Islam-Faridi N, Jones S, Jones-Rhoades M, Jorgensen R, Joshi C, Kangasjärvi J, Karlsson J, Kelleher C, Kirkpatrick R, Kirst M, Kohler A, Kalluri U, Larimer F, Leebens-Mack J, Leplé JC, Locascio P, Lou Y, Lucas S, Martin F, Montanini B, Napoli C, Nelson DR, Nelson C, Nieminen K, Nilsson O, Pereda V, Peter G, Philippe R, Pilate G, Poliakov A, Razumovskaya A, Richardson P, Rinaldi C, Ritland K, Rouzé P, Ryaboy D, Schmutz J, Schrader J, Segerman B, Shin H, Siddiqui A, Sterky F, Terry A, Tsai CJ, Uberbacher E, Unneberg P, Vahala J,Wall K, Wessler S, Yang G, Yin T, Douglas C, Marra M, Sandberg G, Van de Peer Y and Rokhsar D (2006) The genome of black cottonwood, Populus trichocarpa (Torr. & Gray). Science, **313**, 596 – 1604.

∴ Utmazian MNDS, Weishammer G, Vega R and Wenzel WW (2007) Hydroponic screening for metal resistance and accumulation of Cd and zinc in twenty clones of willows and poplars. Environmental Pollution, **148**, 155 – 165.

∴ Van Belleghem F, Cuypers A, Semane B, Smeets K, Vangronsveld J, d'Haen J and Valcke R (2007) Subcellular localization of Cd in roots and leaves of *Arabidopsis thaliana*. New Phytologist, **173**, 3: 495 – 508.

∴ van den Broeck HC, America AHP and Smulders MJM (2008) Staining efficiency of specific proteins depends on the staining method: Wheat gluten proteins. Proteomics, **8**, 1880 – 1884.

∴ van der Lelie D, Schwuchow T, Schwidetzky U, Wuertz S, Baeyens W, Mergeay M and Nies DH (1997) Two component regulatory system involved in transcriptional control of heavy metal homeostasis in *Alcaligenes eutrophus*. Molecular Microbiology, **23**, 493 – 503.

∴ Van Kerkhove E, Pennemand V and Swennen Q (2010) Cd and transport of ions and substances across cell membranes and epithelia. Biometals, **23**, 5: 823 – 855.

∴ Vande Weghe JG and Ow DW (1997) A novel mitochondrial oxidoreductase required for phytochelatin accumulation and Cd tolerance in fission yeast. Journal of Experimental Botany, **48**, 96.

∴ Vandercasteele B, Lauriks R, De Vos B and Tack FMG (2003) Cd and Zn concentration in hybrid poplar foliage and leaf beetles grown on polluted sediment-derived soils. Environmental Monitoring and Assessment, **89**, 263 – 283.

∴ Vander Mijnsbrugge K, Beeckman H, De Rycke R, Van Montagu M, Engler G and Boerjan W (2000a) Phenylcoumaran benzylic ether reductase, a prominent poplar xylem protein, is strongly associated with phenylpropanoid biosynthesis in lignifying cells. *Planta*, **211**, 502 – 509.

∴ Vander Mijnsbrugge K, Meyermans H, Van Montagu M, Bauw G and Boerjan W (2000b) Wood formation in poplar: identification, characterization and seasonal variation of xylem proteins. *Planta*, **210**, 589 – 598.

∴ Vangronsveld J and Clijsters H (1994) Toxic effect of metals. In: Farago ME (ed.) Plants and the chemical elements. VCH Publishers, New York, USA, pp. 149 – 177.

∴ Vangronsveld J, Van Assche F and Clijsters H (1995) Reclamation of a bare industrial area contaminated by non-ferrous metals: in situ metal immobilization and revegetation. *Environmental Pollution*, **87**, 51 – 59.

∴ Vangronsveld J, Colpaert JV and van Tichelen KK (1996) Reclamation of a bare industrial area contaminated by non-ferrous metals: physic-chemical and biological evaluation of the durability of soil treatment and revegetation. *Environmental Pollution*, **94**, 131 – 140.

∴ Vangronsveld J and Cunningham SD (1998) Introduction to the concepts. In: Vangronsveld J and Cunningham SD (eds.) Metal-Contaminated Soils: In-situ Inactivation and Phytorestoration. Springer Verlag, Berlin, Germany, pp. 1 – 15.

∴ Vangronsveld J, Herzig R, Weyens N, Boulet J, Adriaensen K, Ruttens A, Thewys T, Vassilev A, Meers E, Nehnevajova E, van der Lelie D and Mench M (2009) Phytoremediation of contaminated soils and groundwater: lessons from the field. Environmental Science and Pollution Research, **16**, 765 – 794.

∴ Vassilev A, Schwitzguebel JP, Thewys T, van der Lelie D and Vangronsveld J (2004) The use of plants for remediation of metal-contaminated soils. The Scientific World, **4**, 9 – 34.

∴ Vatamaniuk OK, Mari S, Lu YP and Rea PA (2000) Mechanism of heavy metal ion activation of phytochelatin (PC) synthase. The Journal of Biological Chemistry, **275**, 40: 31451 – 31459.

∴ Vaudel M, Sickmann A and Martens L (2010) Peptide and protein quantification. A map of the minefield. Proteomics, **10**, 650 – 670.

∴ Verbruggen N, Hermans C and Schat H (2009) Mechanisms to cope with arsenic or Cd excess in plants. Current Opinion in Plant Biology, **12**, 364 – 372.

∴ Verkleij JAC and Schat H (1990) Mechanisms of metal tolerance in higher plants. In: Shaw AJ (ed.) Evolutionary Aspects of Heavy Metal Tolerance in Plants. CRC Press, Boca Raton, Florida, USA, pp. 179 – 193.

∴ Verret F, Gravot A, Auroy P, Leonhardt N, David P, Nussaume L,Vavasseur A and Richaud P (2004) Overexpression of AtHMA4 enhances root-to-shoot translocation of zinc and Cd and plant metal tolerance. FEBS Letters, **576,** 306 – 312.

∴ Vesey DA (2010) Transport pathways for Cd in the intestine and kidney proximal tubule: focus on the interaction with essential metals. Toxicology Letters, **198**, 1: 13 – 19.

∴ Villiers F, Ducriux C, Hugouvieux V, Jarno N, Ezan E, Garin J, Junot C and Bourguignon J (2011) Inverstigating the plant response to Cd exposure by proteomic and metabolomic approaches. Proteomics, **11**, 1650 – 1663.

∴ Visioli G, Marmiroli M and Marmiroli N (2010) Coupled with mass spectrometry analysis to compare the proteomics response to cadmium stress in plants. Journal of Biomedicine and Biotechnology, **2010**, article ID 567510.

∴ Vizcaíno JA, Côté R, Reisinger F, Foster JM, Mueller M, Rameseder J, Hermjakob H and Martens L (2009) A guide to the Proteomics Identifications Database proteomics data repository. Proteomics, **9**, 4276 - 4283.

∴ Vögeli-Lange R and Wagner GJ (1996) Relationship between Cd, glutathione and Cd-binding peptides (phytochelatins) in leaves of intact tobacco seedlings. Plant Science, **114**, 11 – 18.

∴ Vollenweider P, Menard T, Madeleine S and Günthardt-Goerg (2011) Comparmentation of metals in foliage of *Populus tremula* grown on soils with mixed contamination. I. From tree crown to leaf cell level. Environmental Pollution, **159**, 324 – 336.

∴ von Rozycki T and Nies DH (2009) *Cupriavidus metallidurans*: evolution of a metal resistant bacterium. Antonie van Leeuwenhoek, **96**, 115 – 139.

∴ Waalkens MP (2000) Cd carcinogenesis in review. Journal of Inorganic Chemistry, **79**, 241 – 244.

∴ Wagner GJ (1993) Accumulation of Cd in crop plants and its consequences to human health, Advances in Agronomy, **51**, 173 – 212.

∴ Waisberg M, Joseph P, Hale B and Beyersmann D (2003) Molecular and cellular mechanisms of Cd carcinogenesis. Toxicology, **192**, 95 – 117.

∴ Wang CL, Maratukulam PD, Lum AM, Clark DS and Keasling JD (2000) Metabolic engineering of an aerobic sulfate reduction pathway and its application to precipitation of Cd on the cell surface. Applied and Environmental Microbiology, **66**, 10: 4497 – 4502.

∴ Wang W, Scali M, Vignani R, Spadafora A, Sensi E, Mazzuca S and Cresti M (2003) Protein extractuon for two-dimensional electrophoresis from olive leaf, a plant tissue containing high levels of interfering compounds. Electrophoresis, **24**, 2369 – 2375.

∴ Wang W, Vignani R, Scali M and Cresti M (2006) A universal and rapid protocol for protein extraction from recalcitrant plant tissues for proteomic analysis. Electrophoresis, **27**, 2782 – 2786.

∴ Wassim A, Ichrak BR and Saïda A (2013) Putative role of proteins involved in detoxification of reactive oxygen species in the early response to gravitropic stimulation of poplar stems. *Plant Signalling and Behavior*, **8**, e22411; PMID:23104108.

∴ Watson C, Pulford ID and Riddell-Black D (2003) Screening of willow species for resistance to heavy metals: Comparison of performance in a hydroponics system and field trials. International Journal of Phytoremediation, **5**, 351 – 365.

∴ Weist S, Eravci M, Broedel O, Fuxius S, Eravci S and Baumgartner A (2008) Results and reliability of protein quantification for two-dimensional gel electrophoresis strongly depend on the type of protein sample and the method employed. Proteomics, **8**, 3389 – 3396.

∴ Weist S, Brunkau C, Wittke J, Eravci M, Broedel O, Krause E, Stephanowitz H, Eravci S and Baumgartner A (2010) Effects of thawning, refreezing and storage conditions of tissue samples and protein extracts on 2-DE spot intensity. Proteomics, **10**, 1515 – 1521.

∴ Weyens N, van der Lelie D, Taghavi S, Newman L and Vangronsveld J (2009a) Exploiting plant-microbe partnerships to improve biomass production and remediation. Trends in Biotechnology, **27**, 10: 591 – 598.

∴ Weyens N, van der Lelie D, Artois T, Smeets K, Taghavi S, Newman L, Carleer R and Vangronsveld J (2009b) Bioaugmentation with engineered endophytic bacteria improves contaminant fate in phytoremediation. Environmental Science and Technology, **43**, 24: 9413 – 9418.

∴ Weyens N, Taghavi S, Barac T, van der Lelie D, Boulet J, Artois T, Carleer R and Vangronsveld J (2009c) Bacteria associated with oak and ash on a TCE-contaminated site: characterization of isolates with potential to avoid evapotranspiration. Environmental Science and Pollution Research, **16**, 830 – 843.

∴ Weyens N, van der Lelie D, Taghavi S and Vangronsveld J (2009d) Phytoremediation: plant-endophyte partnerships take the challenge. Current Opinion in Biotechnology, **20**, 248 – 254.

∴ Weyens N, Truyens S, Dupae J, Newman L, Taghavi S, van der Lelie D, Carleer R and Vangronsveld J (2010a) Potential of the TCE-degrading endophyte *Pseudomonas putida* W619-TCE to improve plant growth and reduce TCE phytotoxicity and evapotranspiration in poplar cuttings. Environmental Pollution, **158**, 2915 – 2919.

∴ Weyens N, Croes S, Dupae J, Newman L, van der Lelie D, Carleer R and Vangronsveld J (2010b) Endophytic bacteria improve phytoremediation of Ni and TCE co-contamination. Environmental Pollution, **158**, 2422 – 2427.

∴ Weyens N, Boulet J, Adriaensen D, Timmermans JP, Prinsen E, Van Oevelen S, D'Haen J, Smeets K, van der Lelie D, Taghavi S and Vangronsveld J (2011a) Contrasting colonization and plant growth-promoting capacity between wild type and a gfp-derative of the endophyte *Pseudomonas putida* W619 in hybrid poplar. Plant and Soil, DOI 10.1007/s11104-011-0831-x

∴ Weyens N, Truyens S, Saenen E, Boulet J, Dupae J, Taghavi S, van der Lelie D, Carleer R and Vangronsveld J (2011b) Endophytes and their potential to deal with co-contamination of organic contaminants (toluene) and toxic metals (nickel) during phytoremediation. International Journal of Phytoremediation, **13**, 3: 244 – 255.

∴ WHO (2000) Air quality guidelines for Europe, 2nd Ed. WHO Regional Publications, European Series, No. 91. Regional Office for Europe, Copenhagen, Denmark

∴ Wilkins MR, Appel RD, Van Eyk JE, Chung MCM, Görg A, Hecker M, Huber LA, Langen H, Link AJ, Paik YK, Patterson SD, Pennington SR, Rabilloud T, Simpson RJ, Weiss W and Dunn MJ (2006) Guidelines for the next 10 years of proteomics. Proteomics, **6**, 4 – 8.

∴ Wilm M (2009) Quantitative proteomics in biological research. Proteomics, **9**, 4590 – 4605.

∴ Witters N, Van Slycken S, Ruttens A, Adriaensen K, Meers E, Meirisonne L, Tack FMG, Thewys T, Laes E and Vangronsveld J, 2009, Short-rotation coppice of willow for phytoremediation of a metal-contaminated agricultural area: a sustainability assessment

∴ Wójcik M, Vangronsveld J and Tukiendorf A (2005) Cd tolerance in *Thlaspi caerulescens* I. Growth parameters, metal accumulation and phytochelatin synthesis in response to Cd. Environmental and Experimental Botany, **53**, 151 – 161.

∴ Wu M, Xu MG, Zhang WJ and Wu HW (2012) Effects of soil properties on the stabilization process of cadmium in Cd alone and Cd-Pb contaminated soils. Huan Jing Ke Xue, **33**, 2503 – 2509.

∴ Yang JM, Arnush M, Chen QY, Wu XD, Pang B and Jiang XZ (2003) Cd-induced damage to primary cultures of rat Leydig cells. Reproductive Toxicology, **17**, 553 – 560.

∴ Yeh JI, Chinte U and Du S (2008) Structure of glycerol-3-phosphate dehydrogenase, an essential monotopic membrane enzyme involved in respiration and metabolism. *Proceedings of the National Academy of Science of the USA*¸ **105**, 3280 – 3285.

∴ Yu W, Taylor JA, Davis MT, Bonilla LE, Lee KA, Auger PL, Farnsworth CC, Welcher AA and Patterson SD (2010) Maximizing the sensitivity and reliability of peptide identification in large-scale proteomic experiments by harnessing multiple search engines. Proteomics, **10**, 1172 – 1189.

∴ Zacchini M, Pietrini F, Mugnozza GS, Iori V, Pietrosanti L and Massacci A (2009) Metal tolerance, accumulation and translocation in poplar and willow clones treated with Cd in hydroponics. Water Air and Soil Pollution, **197**, 23 – 34.

∴ Zhang Y, Xiao Y, Du F, Cao L, Dong H and Ren H (2011) Arabidopsis VILLIN4 is involved in root hair growth through regulating actin organization in a Ca2+ dependent manner. *New Phytologist*, **190**, 667 – 682.

∴ Zheng B, Han M, Bernier M and Wen JK (2009) Nuclear actin and actin-binding proteins in the regulation of transcription and gene expression. FEBS Journal, **276**, 2669 – 2685. Doi:10.1111/j.1742-4658.2009.06986.x.

∴ Zhoa L, Sun Y-L, Cui S-X, Chen M, Yang H-M, Liu H-M, Chai T-Y and Huang F (2011) Cd-induced changes in leaf proteome of the hyperaccumulator plant *Phytolacca Americana*. Chemosphere, **85**, 56 – 66.

∴ Zhu YL, Pilon-Smits EHA, Tarun SA, Weber SU, Jouanin L and Terry N (1999) Cd tolerance and accumulation in Indian mustard is enhanced by overexpressing γ-glutamylcysteine synthetase. Plant Physiology, **121**, 1169 – 1177.

∴ Zhuang P, Yang QW, Wang HB and Shu WS (2007) Phytoextraction of heavy metals by eight plant species in the field. Water, Air and Soil Pollution, **184**, 235 – 242.

∴ Zimmer D, Baum C, Leinweber P, Hrynkiewicz K and Meissner R (2009) Associated bacteria increase the phytoextraction of Cd and zinc from a metal-contaminated soil by mycorrhizal willows. International Journal of Phytoremediation, **11**, 2: 200 – 213.

Section VII:

LIST OF PUBLICATIONS AND SCIENTIFIC MANIFESTATIONS

7.1. Scientific publications

2012

∴ Dupae J *et al.*: A comparative study of soluble protein extractions of Populus deltoides x (trichocarpa x deltoides) for two-dimensional gel electrophoresis. Journal of Life Science, **6**, 970 - 977.

∴ Truyens S, Weyens N, Saenen E, Opdenakker K, Cuypers A, Dupae J *et al.*: Inoculation of Arabidopsis thaliana with seed endophytes from Cd selected plants enhances root growth upon exposure to Cd. Environmental Microbiology, submitted

2011

∴ Weyens N, Truyens S, Saenen E, Boulet J, Dupae J *et al.*: Endophytes and their potential to deal with co-contamination of organic contaminants (toluene) and toxic metals (nickel) during phytoremediation. International journal of phytoremediation, 2011, **13** (3): 244 – 255.

∴ Ruttens A, Boulet J, Weyens N, Smeets K, Adriaensen K, Meers E, Van Slycken S, Tack F, Meiresonne L, Thewys T, Witters N, Carleer R, Dupae J and Vangronsveld J.: Short rotation coppice culture of willow and poplar as energy crops on metal contaminated agricultural soils. International Journal of Phytoremediation, 2011, **13**: 194 – 207.

2010

∴ Semane B, Dupae J, *et al.*: Leaf proteome responses of Arabidopsis thaliana exposed to mild cadmium stress. Journal of Plant Physiology, 2010, **167** (4): 247 – 254.

∴ Weyens N, Croes S, Dupae J *et al.*: Endophytic bacteria improve phytoremediation of Ni and TCE co-contamination. Environmental pollution, 2010, **158** (7): 2422 – 2427.

∴ Weyens N, Truyens S, Dupae J *et al.*: Potential of the TCE-degrading endophyte Pseudomonas putida W619-TCE to improve plant growth and reduce TCE phytotoxicity and evapotranspiration in poplar cuttings. Environmental pollution, 2010, **158** (9): 2915 – 2919.

∴ Weyens N, Dupae J *et al.*: Endophytic bacteria strongly reduce TCE evapotranspiration during phytoremediation in the field. Journal of Biotechnology, 2010, **150**: S51 – S51.

∴ Weyens N, Schellingen K, Dupae J *et al.*: Can bacteria associated with willow explain differences in Cd-accumulation capacity between different cultivars? Journal of biotechnology, 2010, **150**: S291 – S292.

7.2. Abstracts

7.2.1. Oral presentations

2011

∴ Weyens N, Dupae J, *et al.*: *Bioaugmentation with Engineered Endophytic Bacteria improves Contaminant Fate in Phytoremediation.* International symposium on Bioremediation and Sustainable environmental technologies. Reno, Nevada, USA. 27 - 30 June 2011

∴ Weyens N, Dupae J, *et al.*: *Bioaugmentation with engineered endophytic bacteria improves contaminant fate in phytoremediation.* 5th European Bioremediation Conference. Chania, Crete, Greece. 4 - 7 July 2011

2010

∴ Weyens N, Dupae J, *et al.*: *Bioaugmentation with engineered endophytic bacteria improves phytoremediation in the field.* SETAC Europe: Annual meeting Science and Technology for Environmental Protection. Seville, Spain. 23 - 27 May 2010

∴ Dupae J *et al.*: *Could equipping plant growth-promoting bacteria (PGPBs) with a metal resistance system enhance phytoextraction of Cd contaminated soils?* International conference on environmental pollution and clean bio/phytoremediation. Pisa, Italy. 16 – 19 June 2010

∴ Weyens N, Dupae J, *et al.*: *Endophytes cut down evapotranspiration during phytoremediation in the field.* International conference on environmental pollution and clean bio/phytoremediation. Pisa, Italy. 16 - 19 june 2010

∴ Vangronsveld J, Truyens S, Dupae J, *et al.*: *Exploiting plant-bacteria partnerships to improve biomass production and remediation of contaminated soils and groundwater.* 12th international symposium on biological nitrogen fixation with non-legumes. Buzios, Rio de Janeiro, Brazil. 3 august 2010

∴ Weyens N, Dupae J, *et al.*: *Endophytic bacteria strongly reduce TCE evapotranspiration during phytoremediation in the field.* 14th international biotechnology symposium and exhibition: Biotechnology for the sustainability of human society. Rimini, Italy. 14 - 18 september 2010

∴ Weyens N, Dupae J,*et al.*: *Endophytic bacteria strongly reduce TCE evapotranspiration during phytoremediation in the field.* Phytotochnologies in the 21st century: challenges after Copenhagen 2009. Remediation-Energy-Health-Sustainability. Parma, Italy. 26-29 september 2010

∴ Weyens N, Dupae J, *et al.*: *Bioaugmentation with engineered endophytic bacteria improves phytoremediation in the field.* Contaminated Site Management in Europe. Gent, Belgium. 19 - 21 October 2010

2009

- Weyens N, Van der Lelie D, Boulet J, Dupae J, *et al.*: *Endophytic bacteria cut down evapotranspiration in the field.* COST 859 - Final international conference. Ascona, Switzerland. 12 - 16 october 2009
- Weyens N, van der Lelie D, Newman L, Boulet J, Dupae J, *et al.*: *Endophytic bacteria cut down evapotranspiration of TCE during phytoremediation in the field.* 6the International Phyto Conference. St. Louis, Missouri, USA. 2 - 4 december 2009

2008

- Weyens N, Barac T, Boulet J, Dupae J, *et al.*: *A role for* plant-associated *bacteria to improve in situ phytoremediation of BTEX and TCE: evidence from 2 field experiments.* Third international meeting on environml biotech and engineering. Palma de Mallorca, Mallorca, Spain. 21 - 25 september 2008
- Weyens N, Barac T, Boulet J, Dupae J, *et al.*: *Modified endophytes for improving phytoremediation of miced contaminations of heavy metals (Ni) and organic contaminants (toluene).* Third international meeting on environml biotech and engineering. Palma de Mallorca, Mallorca, Spain. 21 - 25 september 2008
- Weyens N, Barac T, Boulet J, Dupae J, *et al.*: *A role for* plant-associated *bacteria to improve in situ phytoremediation of BTEX and TCE: evidence from 2 field experiments.* Cost Action 859 - Meeting of Working Group 4 "Integration and application of phytotechnologies". Verneuil en Halatte, France. 15 - 17 october 2008

7.2.2. Poster presentations

2012

- Dupae J *et al.*: Effects of cultivation systems on poplar proteome under control and cadmium-exposed conditions. 9th international IPS conference. Hasselt University, Diepenbeek, Belgium. 11-14 september 2012

2011

- Dupae J *et al.*: *Equipping plant growth-promoting bacteria with a metal resistance system: Effects on metal uptake and translocation.* Final COST meeting – Plant Proteomics in Europe. Dijon, France. 25 – 27 may 2011
- Truyens S, Weyens N, Saenen E, Opdenakker K, Dupae J, *et al.*: *Seed endophytes: essential players in the response of Arabidopsis thaliana after exposure to cadmium?* 1^{1th} International Conference on the Biogeochemistry of Trace Elements. Florence, Italy. 3 - 7 July 2011

2010

- ∴ Dupae J *et al.*: *Leaf and root proteome of soil grown* Cd-exposed *poplar plants.* International Conference. Austria, Vienna. 23 - 26 Februari 2010 (book of abstracts p. 50)
- ∴ Dupae J *et al.*: *Soil grown,* Cd-exposed *poplar plants: a proteome analysis.* System biology and Omic approaches. Namur, Belgium. 5 - 7 May 2010
- ∴ Weyens N, Schellingen K, Dupae J, *et al.*: *Can bacteria associated with willow explain differences in Cd-accumulation capacity between different cultivars?* International conference on environmental pollution and clean bio/phytoremediation. Pisa, Italy. 16 - 19 june 2010
- ∴ Weyens N, Schellingen K, Dupae J, *et al.*: *Can bacteria associated with willow explain differences in Cd-accumulation capacity between different cultivars?* 14th international biotechnology symposium and exhibition: Biotechnology for the sustainability of human society. Rimini, Italy. 14 - 18 september 2010
- ∴ Dupae J, *et al.*: *Can DIGE analysis reveal the underlying mechanisms of enhanced plant growth, metal uptake and translocation caused by PGPBs equipped with a metal resistance system?* Proteomlux 2010. Luxemburg, Luxemburg. 18 - 20 October 2010

2009

- ∴ Dupae J *et al.*: *A comparative study of soluble protein extractions of Populus deltoides x (trichocarpa x deltoides) for two-dimensional gel electrophoresis analysis.* Second meeting of INPAS. Tartu, Estonia. 14 - 17 may 2009 (book of abstracts p.53)
- ∴ Dupae J *et al.*: *Leaf and root proteome of* Cd-exposed *poplar plants: a pot experiment.* COST 859-Final international conference. Ascona, Switzerland. 12 - 16 october 2009 (book of abstracts p.123)

2008

- ∴ Boulet J, Weyens N, Barac T, Dupae J, *et al.*: *Characterisation of soil and plant-associated bacteria on a metal contaminated site.* Third international meeting on environml biotech and engineering. Palma de Mallorca, Mallorca, Spain. 21 - 25 september 2008
- ∴ Dupae J *et al.*: *A comparative study of soluble protein extractions of Populus deltoides x (trichocarpa x deltoides) for two-dimensional gel electrophoresis analysis.* Proteomlux 2008. Luxembourg, Luxembourg. 22 - 24 october 2008 (book of abstracts p.76)
- ∴ Dupae J *et al.*: *A comparative study of soluble protein extractions of Populus deltoides x (trichocarpa x deltoides) for two-dimensional gel electrophoresis.* Third international symposium on proteome analysis. Antwerp, Belgium. 18 – 19 december 2008 (book of abstracts p.79 ISBN: 978-90-5728-110-5)

7.3. Participation at scientific manifestations

2012

∴ 9th international IPS conference. Hasselt University, Diepenbeek, Belgium. 11-14 september 2012

2011

∴ KVCV Workshop proteomics informatics. Louvain, Belgium. 18 april 2011

∴ Final COST meeting – Plant Proteomics in Europe: Where do we stand and where are we heading to? Dijon, France. 25 may 2011 – 27 may 2011.
Poster presentation: Equipping plant growth-promoting bacteria with a metal resistance system: Effects on metal uptake and translocation

2010

∴ Molecular aspects of plant development. Vienna University Economic, Vienna, Austria. 23 – 26 Februari 2010
Poster: Leaf and root proteome of soil grown Cd-exposed *poplar plants.*
Dupae J, Cuypers A, Boulet J, Weyens N, Vangronsveld J

∴ System biology and Omic approaches. Namur, Belgium. 5 - 7 May 2010
Poster: Soil grown, Cd-exposed *poplar plants: a proteome analysis*
Dupae J, Cuypers A, Boulet J, Weyens N, Vangronsveld J

∴ EPCR, International conference on environmental pollution and clean Bio/Phytoremediation. Repubblica Marinara, Pisa, Italy. 16 – 19 June 2010
Oral presentation: Could equipping plant growth-promoting bacteria (PGPBs) with a metal resistance system enhance phytoextraction of Cd contaminated soils?

∴ Proteomic I Course – DIGE Analysis. Kemiskt Biologiskt Centrum (KBC), Umea, Sweden. 13 – 17 september 2010
Course certificate

2009

∴ Plant Abiotic Stress: from signaling to development. Dorpat conference centre, Tartu, Estonia. 14 - 17 May 2009
Poster: A comparative study of protein extraction of Populus deltoides x (trichocarpa x
deltoides) for two dimensional gel electrophoresis analysis
Dupae J, Cuypers A, Boulet J, Weyens N, Vangronsveld J

∴ Phytotechnologies to promote sustainable land use and improve food safety. Centro Stefano Franscini, Monte Verita, Ascona, Switzerland. 12 - 16 October 2009
Poster: Leaf and root proteome of Cd-exposed *poplar plants: a pot experiment*
Dupae J, Cuypers A, Boulet J, Weyens N, Vangronsveld J

2008

- DIGE Meeting. Eindhoven, Netherlands. 15 Oct 2008 - 15 Oct 2008
- Proteomlux 2008. Centre de Recherche Public, Gabrielle lippmann, Luxembourg, Luxembourg. 22 Oct 2008 - 25 Oct 2008
  Poster: A comparative study of protein extraction of Populus deltoides x (trichocarpa x
  deltoides) for two dimensional gel electrophoresis analysis.
  Dupae J, Cuypers A, Boulet J, Weyens N, Vangronsveld J
- Proteome Analysis. Conference centre 'tElzenveld, Antwerp, Belgium. 18 - 19 December 2008
  Poster: A comparative study of protein extractions of Populus deltoides x (trichocarpa x deltoides) for two dimensional gel electrophoresis
  Dupae J, Cuypers A, Boulet J, Weyens N, Vangronsveld J