Republic of Iraq
Ministry of Higher Education
and Scientific Research
Al-Nahrain University
College of Science

The Potential of Some Locally Grown Plants to Genetic Transformation with C*ytochrome p450* and Their Use in Phytoremediation

A Dissertation

Submitted to the College of Science/Al-Nahrain University as a partial fulfilment of the requirements for the award of the Degree of Doctorate of Philosophy in Biotechnology

By

Raghad Salman Mohammed

B.Sc. in Agriculture, Baghdad University, 1996

MSc. Agriculture, Baghdad University, 2002

Supervised by

Dr. Kadhim M. Ibrahim
(Professor)
March
2010

Dr. Norrya A. Ali
(Professor)
Rabi` al-awwal
1431

Supervisors Certification

We, certify that this dissertation entitled "The potential of some locally grown plants to genetic transformation with c*ytochrome p450* and their use in phytoremediation" was prepared by "Raghad Salman Mohammed" under our supervision at the College of Science, Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Doctorate in Biotechnology.

Supervisors

Signature	Signature
Name:	Name:
Dr. Kadhim M. Ibrahim	**Dr. Norrya A. Ali**
Scientific Degree: Professor	Scientific Degree: Professor
Date:	Date:

In view of the available recommendations, I forward this dissertation for debate by the examining committee.

Signature

Name: **Dr. Kadhim M. Ibrahim**

Scientific Degree: **Professor**

Title: **Head of Biotechnology Department**

Date:

Committee's Certification

We, the examining Committee certify that we have read this dissertation entitled "The potential of some locally grown plants to genetic transformation with c*ytochrome p450* and their use in phytoremediation" and examined the student "Raghad Salman Mohammed" in its contents and that in our opinion; it is accepted for the Degree of Doctorate in Biotechnology.

Signature:
Name:
Scientific Degree:
Date:
(Chairman)

Signature:
Name:
Scientific Degree**:**
Date:
(Member)

Signature:
Name:
Scientific Degree:
Date:
(Member)

Signature:
Name:
Scientific Degree**:**
Date:
(Member)

Signature:
Name:
Scientific Degree:
Date:
(Member)

Signature:
Name:
Scientific Degree**:**
Date:
(Member)

Signature:
Name:
Scientific Degree:
Date:
(Member)

I hereby certify upon the decision of the examining committee

Signature:
Name: **Dr. Laith Abdul Aziz Al-Ani**
Scientific Degree: **Assistant professor**
Title: **Dean of the College of Science**
Date:

Summary

To increase phytoremediation potential, *Arabidopsis thaliana* and *Sesbania grandiflora* were genetically engineered with the rabbit cytochrome P450 enzyme. The P450 2E1 enzyme controls the rate-limiting step in the metabolism of multiple environmental pollutants, including Trichloroethylene (TCE), Dichlorodiphenyltrichloroethane (DDT). The percentage of transformation increased at the less diluted cultures of *Agrobacterium tumefaciens*. Similarly, when bacterial concentration decreased, the chance of infected explants increased too and the concentration of *A. tumefaciens* culture was high at the time of infection. The concentration of DDT phytoextracted in this work ranged from 5.7 - 9.3 µg/ml in *S. grandiflora* and *A. thaliana* transgenic plants after 12 days. *S. grandiflora* transgenic lines showed more DDT absorption than *A. thaliana* transgenic plants. Trichloroethane (TCE) results showed a relationship between plant species and TCE metabolism. *A. thaliana* wild type and transgenic plants showed more translocation to Trichloroethanol (TCEOH) compared with Chloral (CH) while reverse thing happen in *S. grandiflora*. *A. thaliana* transgenic lines were more efficient in absorbing TCE in comparison with *S. grandiflora* transgenic lines. The ability of transgenic *S. grandiflora* plants were tested for their potential in remediation of metals (Potassium (K), Calcium (Ca), Manganese (Mn), Iron (Fe), Copper (Cu), Zinc (Zn), Brom (Br) and Lead (Pb)). Clearly, transgenic plants were more efficient achieving in 55, 59, 54, 52, 51, 54, 58 and 51% in comparison with non-transgenic plants. *A. thaliana* and *S. grandiflora* plants strongly expressed the enzyme P450 which is capable of translocating ^{90}Sr, ^{238}U and ^{137}Cs from soils. Results also showed increased translocation (60, 70%) of ^{90}Sr in transgenic *S. grandiflora* and *A. thaliana* respectively in comparison with wild type plants. *A. thaliana* transgenic lines showed an increased ^{238}U

translocation recording 90% and 80% for the F1 and F2 progeny respectively in comparison with wild type plants, while ^{238}U concentration in soil decreased 50% and 60 % for the F1 and F2 progeny respectively.

A. thaliana transgenic lines showed higher ^{137}Cs translocation recording 70% in comparison with the wild type plant, and decreased to 30% in soil planted with transgenic *A. thaliana*.

Acknowledgements

I would like to thank my supervisors, **Dr. Kadhim M. Ibrahim** and **Dr. Norrya A. Ali** for making it possible for me to undertake the project on such an interesting and promising area of research. It is equally important to acknowledge their guidance, enthusiasm and patience during the course of this project.

I am extremely grateful to my 'lab teacher' **Dr. Al-Daoude** (AECS) for his perfect guidance throughout this study. I am indebted to **Dr. S. L. Doty** (University of Washington, USA) and **J. R. Lowty** (UW TechTransfer Invention Licensing) for generously providing the constructed plasmid. I would like also to thank all my colleagues in AL-Nahrain University.

Last but not least, I dedicate this thesis to my husband, Saad for his patience and support during the course of this study and to my children, Hashem and Solafh.

Finally, thanks are due to Dr. Nizar Mir Ali and Dr. Amad AL –Den, at the Atomic Energy Commission of Syria (AECS) for partially sponsoring the project.

For all of them, I said "Thank you all"……

Raghad

List of contents

No.	Subject Title	Page No.
	Summary	
	List of contents	I
	List of tables	VIII
	List of figures	X
	List of abbreviations	xv
	Chapter One	
1	Introduction and Literature review	1
1.1	Introduction	1
1.2	Literature review	2
1.2.1	Hydrocarbon pollutants	2
1.2.1.1	Trichloroethylene	2
1.2.1.2	Dichlorodiphenyltrichloroethane (DDT)	5
1.2.1.3	Toxic metals	7
1.2.2	Phytoremediation	8
1.2.3	Phytoextraction (or) phytoaccumulation	10
1.2.4	Phytodegradation	13
1.2.5	Rhizosphere degradation	18
1.2.6	Rhizofiltration	18
1.2.7	Phytostabilization	18
1.2.8	Phytovolatization	19
1.2.9	Organic pumps	19
1.2.9.1	Cell cultures	19
1.2.9.2	Hairy roots	20

1.2.9.3	Enzymology	22
1.2.9.4	Cytochrome P450-containig monooxygenases	24
1.2.10	Studied plants: *Sesbania grandiflora* and *Arabidopsis thaliana*	27
1.2.10.1	*Sesbania grandiflora*	27
1.2.10.1.1	Plant taxonomy	28
1.2.10.1.2	Plant ecology	29
1.2.10.1.3	Plant uses	30
1.2.10.2	*Arabidopsis thaliana*, the weed that becomes a model plant	31
1.2.11	Phytoremediation with transgenics	33
1.2.11.1	Transgenic phytoremediation of organic pollutants	33
1.2.11.2	Transgenic phytoremediation of metals	36
1.2.11.3	Phytoremediation of radionuclides	38
Chapter Two		
2	Materials and Methods	41
2.1	Materials	41
2.1.1	Apparatus and equipments	41
2.1.2	Chemicals	42
2.2	Methods	43
2.2.1	Sterilization of equipments	43
2.2.2	Plant material	43
2.2.3	Sterilization of explants	44
2.2.4	Preparation of plant tissue culture medium	44
2.2.5	Microorganism	46

2.2.6	Plasmid and bacteria strain for transformation	46
2.2.7	Bacteriological medium	48
2.2.8	Antibiotics	49
2.2.9	Growth and conditions for *A. tumefaciens*	50
2.2.10	Maintenance of isolates	51
2.2.10.1	Maintenance of bacterial isolates	51
2.2.11	Measurement of bacterial growth	52
2.2.12	Sterilization	52
2.2.13	Transformation	52
2.2.13.1	Seeds germination	52
2.2.13.2	Determination of Kanamycin resistance	53
2.2.13.3	Co-cultivation of wounded explants with *A. tumefaciens*	53
2.2.13.4	Plant regeneration	55
2.2.13.5	Establishment of plantlets in the glasshouse	56
2.2.13.6	Transformation of *A. thaliana*	56
2.2.14	Isolation of bacterial DNA	58
2.2.14.1	Isolation of plasmid DNA	58
2.2.14.2	Reagents and chemicals	59
2.2.14.3	Protocol for plasmid DNA isolation	59
2.2.15	Isolation of plant DNA	60
2.2.15.1	Reagents and chemicals	60
2.2.15.2	DNA isolation protocol	61
2.2.15.3	DNA visualization	62
2.2.16	Gel Electrophoresis	62
2.2.16.1	Reagents for Gel Electrophoresis	63
2.2.16.2	Protocol for Gel Electrophoresis	63

2.2.16.2.1	Preparation of an agarose gel	63
2.2.16.2.2	Estimation of DNA concentration	63
2.2.17	Primer design	64
2.2.18	Polymerase chain reaction	64
2.2.18.1	PCR compounds	64
2.2.18.2	PCR protocol	65
2.2.18.3	Analysis of PCR products	66
2.2.19	Western blot analysis	66
2.2.19.1	Reagents of Western blot analysis	66
2.2.19.2	Protocol of Western blot analysis for protein extraction	66
2.2.20	Effect of pollutant compounds	67
2.2.20.1	Effect of Dichlorodiphenyltrichloroethane (DDT)	67
2.2.20.1.1	Germination in different concentration of DDT	67
2.2.20.1.2	Transgenic experiments in the presence of DDT	68
2.2.20.1.3	Extraction of DDT from plant tissues	68
2.2.20.1.4	Calibration curve of DDT	68
2.2.20.2	Effect of Trichloroethane	69
2.2.20.2.1	Germination in different concentration of TCE	69
2.2.20.2.2	Transgenic plants experiments in presence of TCE	70
2.2.20.2.3	Extraction of TCE from plant tissues	70
2.2.20.2.4	Calibration curve of Trichloroethylene	71
2.2.21	GC analysis	71
2.2.22	Effect of metals	72
2.2.22.1	Transgenic experiments in the presence of metals	72
2.2.22.2	Transgenic experiments in contaminated soil	72

2.2.23	Effect of Uranium	73
2.2.23.1	Germination in different concentration of Uranium	74
2.2.23.2	Transgenic plants experiments in presence of Uranium	74
2.2.24	Effect of ^{137}Cesium and ^{90}Strontium	74
2.2.24.1	Transgenic experiments in the presence of ^{137}Cesium and ^{90}Strontium	75
2.2.25	X-ray fluorescence spectrometer (XRF)	75
2.2.26	Gamma analysis	76
2.2.27	Statistical analysis	76
	Chapter Three	
3	Results and Discussion	77
3.1	Molecular analysis	77
3.1.1	*Agrobacterium* regeneration and selection	77
3.1.2	Effect of Kanamycin resistance on plasmid DNA isolation	78
3.1.3	Primer design and PCR amplification	79
3.1.4	Germination of seeds	80
3.1.5	Determination of Kanamycin resistance	81
3.1.6	Transformation	82
3.1.6.1	Inoculation and selective media	82
3.1.6.2	Wounding of explants	84
3.1.6.3	Exposure time of infection	84
3.1.6.4	Callus initiation	85
3.1.6.5	Plant regeneration from callogenisis transgenic cell	87
3.1.6.6	Rooting induction	90
3.1.7	Transformation of *A. thaliana* floral by dipping	91

3.1.7.1	Plant growth stage	93
3.1.7.2	Simplification of the inoculation medium	93
3.1.8	Detection of the transgenic using PCR	94
3.1.9	Western blotting	95
3.2	Effect of pollutants	97
3.2.1	Dichlorodiphenyltrichloroethane (DDT)	97
3.2.1.1	Germination of *S. grandiflora* and *A. thaliana* seeds in the presence of DDT	97
3.2.1.2	Metabolism of DDT	98
3.2.2	Trichloroethylene	103
3.2.2.1	Germination of *S. grandiflora A. thaliana* seeds in the presence of TCE	103
3.2.2.2	Metabolism of TCE	105
3.3	Potassium, Manganese, Copper and Lead accumulation in transgenic plants	109
3.3.1	Potassium accumulation in transgenic plants	109
3.3.2	Manganese accumulation in transgenic plants	111
3.3.3	Copper accumulation in transgenic plants	111
3.3.4	Lead accumulation in transgenic plants	112
3.3.5	Metal effects on plant fresh and dry weight	113
3.4	Effect of contaminated soil with Calcium, Iron, Zinc and Brom	115
3.4.1	Calcium accumulation in transgenic plants	115
3.4.2	Iron accumulation in transgenic plants	115
3.4.3	Zinc accumulation in transgenic plants	117
3.4.4	Brom accumulation in transgenic plants	118

3.5	Effect of radionuclide Uranium, Cesium and Strontium	119
3.5.1	Germination of *A. thaliana* seeds in the presence of Uranium	119
3.5.2	Increased accumulation of Uranium in *A. thaliana*	120
3.5.3	Decreased concentration of Uranium in soil planted with *A. thaliana*	121
3.5.4	Increased translocation of ^{90}Strontium in *A. thaliana* and *S. grandiflora*	122
3.5.5	Increased translocation of ^{137}Cesium in *A. thaliana* and *S. grandiflora*	124

List of Tables

No.	Title of the table	Page No.
2.1	MS medium components used as stock solutions for plant tissue cultures experiments.	45
2.2	LB medium components used as stock solutions for *A. tumefaciens* cultivation.	48
2.3	MG medium components used as stock solutions for A. tumefaciens cultivation.	49
2.4	Stock solution of antibiotics.	49
2.5	Some physicochemical properties of soil used in these experiments.	73
3.1	Effect of BAP and IAA on shoot regeneration from cotyledonary explants of *S. grandiflora* after 4 weeks in culture.	87
3.2	Effect of BAP and NAA on shoot regeneration from cotyledonary explants of *S. grandiflora* after 4 weeks in culture.	87
3.3	Effect of KIN and IAA on shoot regeneration from cotyledonary explants of *S. grandiflora* after 4 weeks in culture.	89
3.4	Effect of KIN and NAA on shoot regeneration from cotyledonary explants of *S. grandiflora* after 4 weeks in culture.	89
3.5	Effect of IBA on rooting of *S. grandiflora* shoots after 6 weeks in culture.	90

3.6	Effect of IAA on rooting of *S. grandiflora* shoots after 6 weeks in culture.	91
3.7	Retention time of DDT, DDD and DDE analyzed by GC-ECD.	97
3.8	DDT uptake, fresh weight and dry weight in transgenic and wild type plants.	101
3.9	Retention time of Trichloroethane, Chloral and Trichloroethanol as determined by GC-ECD.	103
3.10	TCE uptake and fresh weight in transgenic and wild type plant.	107
3.11	Effect of Potassium, Manganese, Copper and Lead mixture at three doses (ppm) on plants fresh and dry weight (g) after 12 days of soil contamination.	113

List of Figures

No.	Title of the figure	Page No.
1.1	Enhancing phytoremediation of organic pollutants using transgenic plants.	14
1.2	Steps involved in phytoremediation.	21
1.3	Microsomal monooxygenase system.	25
1.4	Description of plant metabolism of organic pollutants. Broken arrows represent proposed pathway for glucosylation in Golgi. CT is glutathione-conjugate transporter, AT is ATP-dependent anion transporter, GT is ATP-dependent glucosideconjugate transporter, and VP is vacuolar peptidase.	27
1.5	*Sesbania grandiflora* L. plants at the early stage of growth.	29
1.6	*Arabidopsis thaliana* plant after 30 days from seed sowing (10X).	32
2.1	Plasmid map of pSLD50-6.	47
2.2	Overnight pure culture of *A. tumefaciens* C58Cl grown in LB medium at 28^{o}C and pH 5.7.	50
2.3	Seeds were germinated in MS medium, and incubated at 28^{o}C.	53
2.4	*Sesbania grandiflora* transformation process.	54
2.5	Shoot regeneration and root induction on MS medium containing 50 mg/l Kanamycin.	55
2.6	Plantlets were established in a glasshouse (A) and transferred to a 50:50 peat: soil mixture (B,C) or 50 ml flasks (D) containing half strength liquid MS medium supplemented with Kanamycin 50 mg/l.	56
2.7	*A. thaliana* florets were dipped with aliquots containing *A. tumefaciens*	58
2.8	Calibration curve of DDT. R^2 value is 0.971.	69

2.9	Calibration curve of TCE. R^2 value is 0.953.	71
3.1	*A. tumefaciens* colonies stained with Gram stain 250 X under light microscope. The bacteria was grown on LB medium containing 50 mg/l Kanamycin and 100 mg/l Carbincillin for two days at 25^{o}C.	78
3.2	Agarose (2%) gel electrophoresis of total DNA extracted from *A. tumefaciens* (70 v, 60 minutes).	79
3.3	Agarose (2%) gel electrophoresis of PCR amplicons using CYP2E1 specific primers (70 v, 60 minutes).	80
3.4	*S. grandiflora* and *A. thaliana* seeds germinated on half strength MS medium solidified with 7 g/l agar or on moistened sterile filters paper. Both plants are identical in their % germination.	81
3.5	*S. grandiflora* and *A. thaliana* seeds germinated on half strength MS medium containing different concentrations of Kanamycin for 10 days. Both plants are identical in their % germination.	82
3.6	*S. grandiflora* explants appeared watery (A) and viscous (B) after 2-4 days of inoculation with *A. tumefaciens* on MG/L medium.	83
3.7	*S. grandiflora* explants appeared viscous and contracted after 35 (A) and 40 (B) minutes of infection with *A. tumefaciens*.	85
3.8	Effect of Carbincillin (400 mg/l) and Kanamycin (50 mg/l) on transformed (A) and non transformed (B) explants cultured on MS medium.	85
3.9	Growth and elongation of embryogenic calli on MS medium containing 400 mg/l Carbincillin and 50 mg/l Kanamycin.	86
3.10	*A. thaliana* growth stages with floral dripping method.	92

3.11	Agarose (2%) gel electrophoresis of PCR amplicons using CYP2E1 specific primer (70 v, 60 minutes). Lane 1: DNA Ladder (Band of around 2500 – 200 bp). Ninety six DNA samples were extracted and tested for the presence of CYP2E1 gene (a band of ~410 bp was seen).	94
3.12	Agarose (2%) gel electrophoresis of PCR amplicons using CYP2E1 specific primer (70 v, 60 minutes), DNA Ladder band of around 2500 – 200 bp. Twenty four DNA samples were seen with bands around 410 bp (CYP2E1 gene).	95
3.13	Detection of CYP2E1 in transgenic *A. thaliana* and *S. grandiflora* plants using western blotting technique.	96
3.14	Germination percentages of *S. grandiflora* and *A. thaliana* seeds after 15 days of sowing on different DDT concentrations. Both plants behaved similarly.	98
3.15	Concentration of DDT and its metabolism to DDD, DDE in *A. thaliana* plants and culturing medium after 12 days.	99
3.16	Concentration of DDT and its metabolism to DDD, DDE in *S. grandiflora* plants and culturing medium after 12 days.	99
3.17	Germination percentages of *S. grandiflora* and *A. thaliana* seeds after 15 days of sowing on different TCE concentrations. Both plants behaved similarly	104
3.18	TCE Concentration (g/l) and its metabolism to Chloral, and Trichloroethanol in transgenic (GM) and wild type (WT) *A. thaliana* and *S. grandiflora* plants after 7days.	106
3.19	Metal accumulation in *S. grandiflora* transgenic plants and residual concentrations in soil after 12 days of planting.	110
3.20	Metal accumulation in *A. thaliana* transgenic plants and residual concentrations in soil after 12 days of planting.	110

3.21	Metal accumulation in *S. grandiflora* transgenic plants after 5, 10 and 15 days of planting and in soils planted with *S. grandiflora* transgenic plants.	116
3.22	Metal accumulation in *A. thaliana* transgenic plants after 5, 10 and 15 days of planting and in soils planted with *A. thaliana* transgenic plants.	116
3.23	Germination percentages of *A. thaliana* seeds at 7 and 15 days after sowing on different ^{238}U concentrations (0, 25 and 50mM).	119
3.24	Concentration of ^{238}**U** in transgenic *A. thaliana* F1 (F1 Ara.GM) and F2 (F2 Ara.GM) progeny and in wild type plants (Ara.Wt) after 12 days of planting.	120
3.25	Concentration of ^{238}U in soil planted with transgenic *A. thaliana* F1 and F2 progeny and wild type compared with the initial concentration added to the soil after 12 days of planting.	121
3.26	Concentration of ^{90}Sr in *A. thaliana* and *S. grandiflora* transgenic and wild type plants (control) after 7 days.	123
3.27	Concentration of ^{90}Sr in soils planted with transgenic *A. thaliana* and *S. grandiflora* plants and wild type plants (control) after 7 days.	123
3.28	Concentration of ^{137}Cs in transgenic *A. thaliana* and *S. grandiflora* plants and wild type plants (control) after 7 days.	123
3.29	Concentration of ^{137}Cs in soils planted with transgenic *A. thaliana* and *S. grandiflora* plants and wild type plants (control) after 7 days.	125
3.30	Transgenic and wild type (control) *Arabidopsis* plants grown on soil contaminated with 17600 Bq/g ^{137}Cs	126
3.31	Transgenic *S. grandiflora* plants grown on soil contaminated	126

	with 17600 Bq/g ^{137}Cs	
3.32	*S. grandiflora* wild type (control) grown on soil contaminated with 17600 Bq/g ^{137}Cs	127

List of abbreviations

1,2-DCA	1,2-dichloroethane
16S rRNA	16S Ribosomal RNA
2,4-D	2,4-dichlorophenoxyacetate
As	Arsenic
ATP	Adenosine triphosphate
Br	Bromine
Ca	Calcium
CaMV	Cauliflower mossic virus
Cd	Cadmium
CF	Chloroform
CH	Chloral hydrate
Co	Cobalt
Cr	Chromium
Cs	Cesium
CT	Carbon tetrachloride
Cu	Cupper
DCE	Dichloroethene
DDD	1,1,1-Trichloro-2,2-bis (*4*-chlorophenyl) ethane
DDE	1,1,1-Trichloro-2,2-bis (*p*-chlorophenyl) ethylene
DDT	1,1,1-Trichloro-2,2-bis(*p* -chlorophenyl)ethane
DU	Depleted Uranium
DW	Dry weight

List of Abbreviations

ECD	Electron capture detector
Fe	Ferro's
FW	Fresh weight
GC	Gas chromatography
GDN	Glycerol dinitrate
GMN	Glycerol mononitrate
GTN	Glycerol trinitrate
Gus	β-Glucronidaseer
Hg	Mercury
K	Potassium
LAC1	Lactase 1
mM	Millimolar
Mn	Manganese
Mo	Molybdenum
MTBE	Methyl *tert*-butyl ether
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
ng/l	part per trillion (Nanogram/L)
Ni	Nickel
PAHs	Polycyclic aromatic hydrocarbons
Pb	Lead
PCBs	Polychlorinated biphenyls
PCE	Tetrachloroethylene (Perchlorethylene)
PCP	Pentachlorophenol

List of Abbreviations

PCR	Polymerase chain reaction
ppb	Part per billion (1 ppb = .001 ppm; 1 ppb = 1 μg/l)
ppm	Part per million (1 ppm = 1000 ppb; 1 ppm = 1 mg/l)
Rb	Rubidium
RDX	Royal demolition explosive
SDS	Sodium dodecyl sulphate
SDS-PAGE	Polyacrylamide gel electrophoresis in the presence of SDS
SDW	Sterile distilled water
Sr	Strontium
TCAA	Trichloroacetic Acid
TCE	Trichloroethylene
TCEOH	Trichloroethane
TNT	Trinitrotoluene
U	Uranium
$UO_2 (NO_3)_2$	Nitrate uranium oxide
VC	Vinyl chloride
VOC	Volatile organic compounds
WT	Wild-type
Zn	Zinc

Chapter One
Introduction and Literature Review

1 Introduction and Literature review

1.1 Introduction

Many plant species have an inherent ability to metabolize and detoxify a variety of environmental pollutants. The genus *Sesbania* belongs to the family fabaceae, which includes important plants that are used for treatment of contaminated soils. It is known for exceptionally fast growth rates as well as a very high affinity for association with nitrogen fixing bacteria and has several potential uses including forage, fuelwood, pulpwood, fences, medicines, shade and for soil improvement (Onim and Dzowela, 2006).

The effectiveness of phytoremediation can be greatly enhanced by introducing genes known to be involved in metabolism, accumulation and detoxification of pollutants transferred from other organisms. Transgenic plants expressing cytochrome *P450 2E1* genes have enhanced phytoremediation of organic pollutants. Development of transgenic plants for enhanced phytoremediation of metals has also been successful including plants that developed to detoxify and remove Pb, Br, Zn, Mn, Fe, Cu, Sr, U, Cs, DDT, and TCE (Doty *et al*., 2002).

Phytoremediation is the use of plants and their associated microorganisms in optimized agronomical conditions to remove, or render harmless contaminants, including organic compounds, emission radionuclides, and toxic metals. It is a technology that aims at providing a cheap, soft and safe treatment applicable to contaminated sites. This is based on many considerations, short-term effectiveness, and reduction in the toxicity,

mobility, or volume of toxic elements concerned, feasibility, cost, and overall benefit to the environment (Meagher and Heaton, 2005).

Due to the importance of enhancing transgenic plants for phytoremediation purposes, the aim of this study was:

Transformation of *Sesbania grandiflora* and *Arabidopsis thaliana* with the gene *cytochrome P450 2E1* to enhance their phytoremediation capabilities for detoxification of several wide spread and dangerous pollutants in soil.

1.2 Literature review

1.2.1 Hydrocarbon pollutants

1.2.1.1 Trichloroethylene (TCE)

Trichloroethylene (1, 1, 2-trichloroethene) is a clear, colourless, non-viscous liquid, slightly sweet odour. It is an unsaturated, chlorinated aliphatic compound (chemical formula C_2HCl_3) with a low molecular weight (131.4 $g \cdot mole^{-1}$), and a powerful solvent for a large number of natural and synthetic substances. Trichloroethylene is a volatile liquid at room temperature (melting point -73°C, boiling point 86.7°C) with high density (1.46 g/ml at 20°C) and low surface tension (0.029 n/m) (Chappell, 1998).

High levels of trichloroethylene in groundwater are associated with leaching from specific sources, such as landfill waste disposal sites. Trichloroethylene is one of the most frequently observed volatile organic compound found in municipal sewage entering public treatment works in the U.S. (Kassel *et al.*, 2002).

Generally, the majority of TCE released to soil surfaces volatilizes to the atmosphere. However, significant accumulation of the chemical in saturated and unsaturated zones may result where TCE penetrates the surface before evaporation (Schwille, 1988). In most cases, TCE enters the soil media as an undiluted solution from spills or leaking storage tanks, as leachate from landfill sites, or by wet deposition in rain and snow from the atmosphere (Muraoka and Hirata, 1988). It is highly mobile in the subsurface environment and susceptible to leaching (Newman *et al.,* 1997). The half-life of TCE in soil as determined by the level two fugacity model is 1700 hrs (Mackay *et al.,* 1993).

Trichloroethylene metabolism occurs primarily in the liver (Health Canada, 2004). It is metabolized by two main pathways: oxidation by cytochrome P450 and conjugation with glutathione by glutathione-S-transferases. Exposure to trichloroethylene results in exposure of tissues to a complex mixture of metabolites (USEPA, 2001).

The initial step in the oxidative metabolic pathway is thought to be the formation of an unstable epoxide (trichloroethylene oxide). The predominant pathway is then spontaneous rearrangement to chloral (2, 2, 2-trichloroacetaldehyde; CCl_3-CHO), followed by hydration to chloral hydrate (2, 2, 2-trichloro-1, 1-ethanediol; CCl_3-CH $(OH)_2$). Chloral hydrate is then metabolized to trichloroacetic acid (CCl_3-COOH) and other metabolites. Trichloroacetic acid is the primary trichloroethylene metabolite in blood (Health Canada, 2004).

Ma and Burken (2002; 2003) demonstrated that TCE is taken up by hybrid poplars and volatilizes to the atmosphere. The diffusion of TCE along the transpiration pathways is the dominant process, although volatilization also occurs through the stems and leaves. Laboratory and field studies concluded that TCE transpiration rates decreased with elevation (tree height) and in the radial direction, providing fundamental evidence for diffusion. Poplar cuttings showed no signs of toxicity or inhibition in these short-term experiments at a concentration up to 50 ppm. No leaf wilting, chlorosis, or water usage reduction was observed. Partitioning coefficients of TCE between water, air, and biomass were determined by the physicochemical characteristics of the contaminant, such as Henry's law constant and vapour pressure.

Four different P450 isoforms have been identified that play a role in TCE metabolism: CYP1A1/2, CYP2B1/2, CYP2C11/6, and CYP2E1. Of these isoforms, CYP2E1 appears to be the major form with the highest affinity for TCE (Guengerich, 1991), although considerable variability can exist in the relative roles of different isoforms, depending on physiological state and on the presence of other drugs or inducing agents. Furthermore, as discussed below, most of the work on oxidative metabolism of TCE has been done in the liver. Consequently, it was not known if isoform specificity for TCE was the same in other tissues. Additionally, P450 isoform specificity in different animal species has not been thoroughly investigated. Differences among animal species in isoform content and specificity may play a role in the observed differences in metabolism and toxicity (Doty *et al.,* 2007).

1.2.1.2 Dichlorodiphenyltrichloroethane (DDT)

DDT was an organochlorine insecticide used mainly to control mosquito-borne malaria. It is used on crops and has generally been replaced by less persistent insecticides. It was extensively used during the Second World War among Allied troops and certain civilian populations to control insect typhus and malaria vectors, and was then extensively used as an agricultural insecticide after 1945. DDT was banned for use in Sweden in 1970 and in the United States in 1972 (WHO, 1999).

Technical grade DDT is actually a mixture of three isomers of DDT, principally the p,p'-DDT isomer (85%), with the o,p'-DDT and o,o'-DDT isomers typically present in much lesser amounts (WHO, 1999).

It was thought that inhalation or exposure to DDT will not result in significant absorption through the lung alveoli (tiny gas-exchange sacs) but rather that it is probably trapped in mucous secretions and swallowed by exposed individuals following the tracheo-bronchial clearance of secretions by the cilia (Rsoc, 1999).

DDT is very highly persistent in the environment, with a reported half life between 2-15 years and immobile in most soils. Routes of loss and degradation include runoff, volatilization, photolysis and biodegradation (aerobic and anaerobic). These processes generally occur only very slowly. Breakdown products in the soil environment DDE and DDD, which are also highly persistent and have similar chemical and physical properties (Osta, 2004).

Due to its extremely low solubility in water, DDT will be retained to a greater degree by soils and soil fractions with higher proportions of soil organic matter. Generally DDT is tightly absorbed by soil organic matter, but it has been detected in many locations in soil and groundwater. It may eventually leach into groundwater, especially in soils with little soil organic matter (Njoroge and Bussmann, 2008).

Residues at the surface of the soil were much more likely to be broken down or otherwise dissipated than those below several inches. Studies in Arizona have shown that volatilization losses may be significant and rapid in soils with very low organic matter content (desert) and high irradiance of sunlight, with volatilization losses reported as high as 50% in 5 months. In other soils (Hood River and Medford) this rate may be as low as 17- 18% over 5 years. Volatilization loss will vary with the amount of DDT applied, proportion of soil organic matter, proximity to soil-air interface and the amount of sunlight (Stode *et al.,* 2006).

DDT may reach surface waters primarily by runoff, atmospheric transport, drift, or by direct application (e.g. to control mosquito-borne malaria). The reported half-life for DDT in the water environment is 56 days in lake water and approximately 28 days in river water. The main pathways for loss are volatilization, photodegradation, adsorption to water-borne particulates and sedimentation. Aquatic organisms also readily take up and store DDT and its metabolites. Field and laboratory studies in the United Kingdom demonstrated that very little breakdown of DDT occurred in estuary sediments over the course of 46 days (Osta, 2007).

Noting that DDT-metabolizing and cypermethrin-metabolizing P450s have already been identified in the CYP6 family of dipterans (*Drosophila melanogaster* and *Musca domestica*) and lepidopterans (*Helicoverpa zea*). It is important to determine whether any of CYP6 family transcripts can be expressed at higher levels in DDT-resistant strains that have potential to code for a P450 and capable of metabolizing DDT (Scott, 1999).

Comparisons indicated that, although CYP6 family share high primary sequence identity, their predicted substrate cavities were dramatically different, with CYP6Z1 predicted to metabolize DDT and CYP6Z2 predicted not to bind this insecticide. Subsequent biochemical characterizations supported these predictions and identified CYP6Z1 as a potential target for the design of inhibitors capable of reducing *Anopheles gambiae* resistance to DDT and other insecticides (Rupasinghe *et al.,* 2007).

1.2.1.3 Toxic metals

Geological and anthropogenic activities are two sources of heavy metal contamination (Dembitsky, 2003). Volcanic activities, industrial effluents, fuel production, mining, smelting processes, military operations, utilization of agricultural chemicals, and manufacturing products release enormous amount of heavy metals into the soil, water and air. Severe contamination of heavy metals in soils may cause a variety of environmental problems, including groundwater contamination and toxicity to plants, animals, and humans (Jianwei *et al.,* 1997).

Toxic metals interfer with biochemical and homeostatic processes in the cell through the production of free radicals. The biological consequence of each metal depends on the target accumulation organ, the particular chemical pathway

that disrupts the chemical form of the metal, and its oxidation state (McIntyre, 2003). Kidney dysfunction is a common effect of exposure to As, Cd, Pb, Hg and U (Schnoor *et al*., 1995) while chromosomal damage and cancer have been shown in humans exposed to Cd and Pb (Tsao, 2003). Neurological, hematological, immune effects have also been shown in humans exposed to lead (Cunningham *et al.,* 1995).

As a consequences of the past war activities a large area in Kosovo and some locations in South Serbia (Yugoslavia), and two wars in Iraq caused contamination by depleted uranium (DU) and toxic heavy metals. There is an urgent need for remediation of this contamination in order to prevent its possible long-term effects not only on the population in the contaminated regions but also on the neighboring countries. A different phytoremediation approach is known as phytoextraction. Phytoextraction, also known as phytoacumulation, phytoabsortion or phytosequestration, was developed specifically for inorganic pollutants such as Ag, Cd, Co, Cr, Cu, Hg, Mn, Mo, Ni, Pb, and Zn, metalloids (As, Se), radionuclides (^{90}Sr, ^{137}Cs, ^{243}U, and ^{238}U) and nonmetals (Schnoor *et al*., 1995). In this process, the removal of contaminants is achieved through the root network and the accumulation potential into the plant biomass. After sequestration, the biomass is harvested to complete the extraction of contaminants from the environment (McIntyre, 2003).

1.2.2 Phytoremediation

Phytoremediation is utilizing plants to treat contaminated sites. It takes the advantage of plants natural ability to extract chemicals from water, soil, and air. Furthermore, phytoremediation has been used to treat a variety of

pollutants including metals, petroleum, solvents, explosives, polycyclic aromatic hydrocarbons, and other organic contaminants (Doty *et al*., 2007).

Plants supply nutrients for rhizospheric bacteria that may aid in remediation of the pollutants and act as soil stabilizers, minimizing the amount of contaminated dust that could leave the site and enter the surrounding neighborhoods. Unlike bioremediation, phytoremediation is visible where plants condition can be visually monitored, and samples of plant tissue can be tested for the presence of pollutants over time. Moreover, phytoremediation includes the possibility of a useful product such as wood, pulp, or bioenergy that could help finance the clean up (Stanton *et al*., 2002).

Phytoremediation also provides wildlife habitat where poplar (*Populus spp.*) tree plantations can harbor an abundance of birds and small mammals (Moser *et al*., 2002), and willow (*Salix spp.)* can provide the stopover sites for 60 migrating bird species (Kuzovkina and Quigley, 2005).

Remediation of metals presents a distinct challenge because the pollutants cannot be metabolized but must instead be translocated to the foliage where they are more easily removed by harvesting the upper parts of the plant, or are volatilized such as in the case of mercury. Phytoextraction, the removal of contaminants from the soil and translocation to the foliage, is an effective means for remediating a site because it reduces the overall mass to be treated from tons of widespread contaminated soil to plant tissue that can be dried to a small volume. To be effective, the concentration of the metal in the harvestable part of the plants must be higher than the concentration in the soil so that the volume of the hazardous plant waste is less than the volume of the

contaminated soil. Unlike engineering methods that would remove the fertile topsoil, phytoremediation would not reduce the fertility of the site (Robinson *et al*., 2000).

The Environmental Protection Agency defines phytoremediation as using plants to decontaminate any medium from environmental pollution, in whole or in part. Like other remediation methods, phytoremediation may lead to several different acceptable outcomes and has several distinct modes of action such as phytostabilization, immobilization, accumulation, volatilization, phytodegradation, and rhizodegradation. In immobilization or stabilization, the pollutant is adsorbed to the plant roots and is prevented from migrating, or, from reacting with root exudates, forms an insoluble precipitate that is no longer bioavailable and does not enter ground water. Immobilization prevents the contaminant from having a deleterious effect on the ecosystem, but, since it does not destroy the contaminant, care must be taken to ensure that the pollutant does not mobilize again at a later date due to environmental changes or the death of the plant (Schnoor *et al.*, 1995).

1.2.3 Phytoextraction (or) phytoaccumulation

Phytoextraction also known as phytoaccumulation, the chemical of concern is taken up into the plant, thus the contaminant is removed from the original matrix and the plant material is harvested and usually landfilled. The main advantage of this technique is the ability to concentrate the pollutant and to prevent it from extended interaction with the ecosystem. However, the contaminant is not destroyed, and a suitable disposal method and location must be found for the contaminated plant tissue (Salt and Kramer, 1998). The technique is generally used for removing metals like nickel, zinc, copper, lead,

chromium and cadmium. Since plant roots take and store them in the harvestable regions (leaves and stems) (Doty *et al*., 2000).

Some plants are defined as metal hyperaccumulators. They concentrate metals up to a level of 0.1% for nickel, cobalt, copper, and lead, 1% for zinc, and 0.01% for cadmium (Baker, 1999). Chinese brake fern (*Pteris vittata*) efficiently hyperaccumulates arsenic in its fronds which can be effectively harvested. Arsenic is a lethal poison that is released into the environment from natural processes and through the use of arsenic-based chemicals. The fern can effectively removes this metal from soil. For example, in soil contaminated with arsenic at a concentration of 97 ppm, the older fronds of the fern contain arsenic concentrations of up to 3894 μg per gram of tissue. Less than 168 ppm arsenic was found in the root tissue. More than 95% of the arsenic removed from soil by the fern was translocated to the aboveground biomass. Unfortunately, *P. vittata* species grows well only in warm, humid environments with mild winters (Zhang *et al*., 2002).

Another hyperaccumulator is *Thlaspi caerulescens*, which concentrates cadmium, a highly toxic and probably carcinogenic metal (Vido, 2001), in the above-ground tissues at concentrations 1000 times higher than the normal toxic concentration of only 1 ppm. Plants were exposed to 2000 ppm cadmium and accumulated 1140 ppm (Brown *et al*., 1995).

In another work using *Agrobacterium rhizogenes*-induced root cultures of *T. caerulesens*, up to 600 ppm cadmium of dry weight of roots was accumulated (Nedelkoska and Doran, 2000). The mechanism of cadmium

uptake and zinc by this member of the Brassicaceae family has been well studied and involves a highly expressed metal transporter (Pence *et al*., 2000).

The transporter gene, *ZNT1*, encodes a high-affinity zinc/low-affinity cadmium transporter, as demonstrated in yeast. The zinc/cadmium pumping ATPase was recently purified directly from *T. caerulescens* and was shown to transport both zinc and cadmium (Parameswaran *et al*., 2007).

Although the research on these hyperaccumulating species is promising, the species themselves are too small and slow-growing for particular phytoremediation applications (Ebbs *et al*., 1997).

Therefore, high-biomass crops such as poplar and willow are being studied for phytoremediation of metals. They are not hyperaccumulators as they do not concentrate metals to high concentrations, but, because of their greater biomass and deep root systems, they are also effective remediators of metal contamination.

In a review by Dickinson and Pulford (2005), willow was specifically suggested for phytoremediation of heavy-metal contaminated lands because the method requires the ability of a plant to re-grow readily after its shoots have been harvested, a distinctive trait of willow.

The 'bioconcentration factor' (BCF) refers to the metal concentration in plant tissues relative to the metal concentration in the substrate, and a value greater than 1 means that the plant actively concentrates the metal. Furthermore, BCF was reported to vary widely among different willow species, from as low as 0.05–16.8 in woody stems up to 27.9 in foliage (Dickinson and Pulford, 2005). Given the substantial genetic diversity of

willow, with over 450 *Salix* species, this variability is not too surprising. Willow (*Salix matsudana* × *Salix alba* NZ1040) grown in soil contaminated with cadmium at concentrations commonly found in agricultural sites fertilized with cadmium-containing fertilizers accumulated cadmium in the above-ground tissues with BCF of 10 (Robinson *et al*., 2000).

1.2.4 Phytodegradation

Phytodegradation is the uptake and metabolism of a contaminant by a plant where it is converted into less toxic forms. It is a permanent solution for organic chemical contamination, and has the advantage that the site no longer needs monitoring after target concentrations are reached.

Also phytodegradation involves the metabolic degradation of organic pollutants, in this process; plants break down the pollutant through either internal or secreted enzymes. Phytodegradation of chlorinated hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), and explosives have been well studied (Bruning and Bolt, 2000).

Hybrid poplars (*Populus trichocarpa* × *Populus deltoides*) take up and degrade TCE, producing the same TCE metabolites as mammals (Newman *et al*., 1997; Gordon *et al*., 1998). In a controlled study, hybrid poplar removed over 99% of the added TCE (Newman *et al*., 1999). When these poplar culture cells were dosed with TCE, the same metabolites were seen as those in the whole plant (Newman *et al*., 1997; Shang *et al*., 2001; Shang and Gordon, 2002). Experiments with both poplar culture cells and whole plants demonstrated that trichloroethanol, is glycosylated, as happens in mammalian systems (Shang *et al*., 2001). Other plant species are also able to take up and

metabolize TCE, such as the tropical leguminous tree *Leucaena leucocephala* (Doty *et al*., 2003) and sweet potato plants *Ipomoea batatas* (Doty, 2008).

The phytotoxic effects of trinitrotoluene (TNT) and Royal Demolition Explosive (RDX; hexahydro-1,3,5-trinitro-1,3,5-triazine) are overcome by expressing the bacterial genes *xpl* A/B and *nfsI*, allowing the plant to more effectively remove these pollutants (Figure 1.1). Trichloroethylene (TCE) and other small volatile chemicals are more readily taken up and degraded by transgenic plants expressing mammalian *CYP2E1*, removal of volatile because of their carcinogenic properties and prevalence.

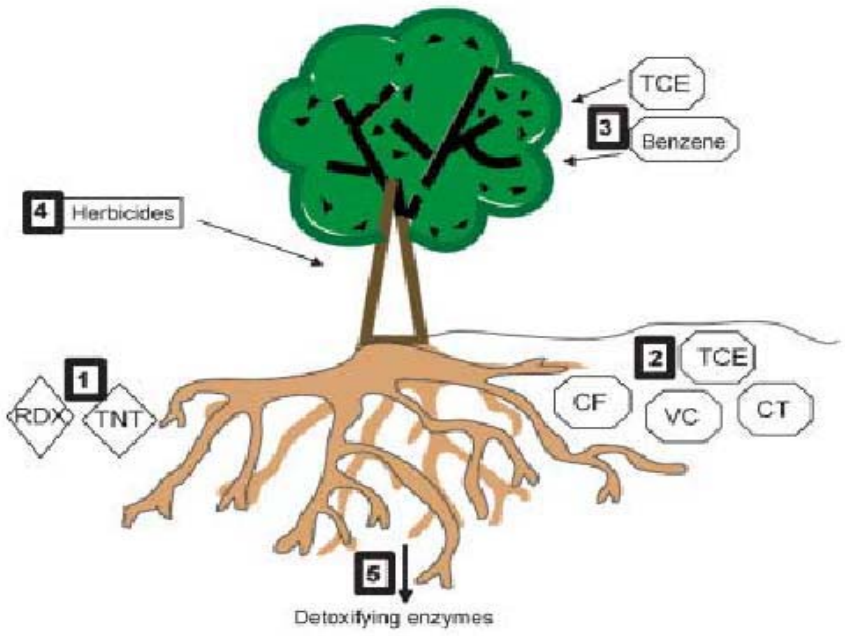

Figure (1.1): Enhancing phytoremediation of organic pollutants using transgenic plants (Wang *et al*., 2002). CT: Carbon tetrachloride, CF: Chloroform, VC: Vinyl chloride, TCE: Trichloroethylene, TNT: Trinitrotoluene, RDX: Royal Demolition Explosive.

TCE and benzene from the air was also enhanced by *CYP2E1* in transgenic plants. Expression of mammalian *CYP2B6* or gamma-glutamylcysteine synthetase helps plants to degrade a variety of herbicides. Secretion of the detoxifying enzymes such as lactase 1 (LAC1) or haloalkane dehalogenase enabled transgenic plants to degrade phytotoxic pollutants without taking them up. Carbon tetrachloride's (CT), chloroform; vinyl chloride (VC). Anaerobic degradation of CT by soil microbes can result in carcinogenic chloroform production; by contrast, plants are capable of metabolizing CT aerobically using a cytochrome P450 enzyme (Wang *et al*., 2002).

In a controlled field study in which poplar trees were watered with 12–15 mg l^{-1} CT over a 6-year period, CT was taken up and dechlorinated (Wang *et al*., 2004). There was no significant evapotranspiration of CT, nor increased accumulation of chloride ion in the dosed trees compared with untreated ones, but chloride ions had built up in the root zone. Because soil microbes from the site did not dechlorinate CT, the authors concluded that the trees had taken up and dechlorinated CT, and then exported excess chloride ions into the soil. Poplar trees are also effective in remediating perchloroethylene. Nearly all of this pollutant was removed and metabolized, with over 95% of the chlorine recovered as free innocuous chloride, showing effective dechlorination of the perchloroethylene (Doty, 2008).

The USEPA lists sixteen PAHs as priority pollutants. Phytodegradation of PAHs occurs since they are lipophilic, adsorption to root surfaces may be an important first step in phytoremediation (Burken and Schnoor, 1998; Schwab *et al*., 1998).

Research by several laboratories has demonstrated that there are wide differences in the abilities of different plant species to reduce PAH concentrations (Trenck and Sandermann, 1979), and that plants themselves can degrade PAHs (Harms, 1996).

Wittig *et al*. (2003) conducted a three-part investigation in the use of poplar cuttings for PAH removal. *Populus nigra* cuttings grown in containers of sand irrigated with nutrient solution containing PAHs caused a reduction in the amounts of a range of PAHs, including anthracene, phenanthrene, pyrene, fluoranthene, chrysene, and benzo [a] pyrene. An extensive field study was conducted using poplar trees to reduce the PAH concentration in groundwater (Widdowson *et al*., 2005).

Another important class of environmental pollutants for which plants can be used for remediation is explosives including trinitrotoluene (TNT) and Royal Demolition Explosive (RDX; hexahydro-1,3,5-trinitro-1,3,5-triazine). TNT is toxic to humans, causing plastic anemia, hepatitis and affects the central nervous system (Rosenblatt, 1980).

More than 100 military bases and explosives-manufacturing facilities in the USA are contaminated with these chemicals. The groundwater at these sites is contaminated, increasing the hazard that the health risk will spread beyond the military bases (Rivera *et al*., 1998).

Research with aquatic plants demonstrated that TNT could be metabolized in the absence of microorganisms (Hannink *et al*., 2002). Both poplar and willow have been used for inmunitions remediation research. Hybrid poplar (*P. deltoides* × *P. nigra*) was able to take up TNT from

hydroponic solution, but the trees only translocated 10% of it to the foliage (Thompson *et al*., 1998).

In a study, comparing phytoremediation of TNT by hybrid willow (*Salix* clone EW-20) and Norway spruce (*Picea abies*), it was shown that both trees readily metabolize TNT (Schoenmuth and Pestemer, 2004). The trees were exposed to 5.2 mg TNT per kg soil and two months later 3–14% of the radiolabeled TNT was translocated to the aboveground tissues. Poplar tissue cultures and leaf extracts exposed to 20 mg l^{-1} of this explosive mineralized 17% of the RDX to carbon dioxide when exposed to light (Aken *et al*., 2004). RDX uptake was also studied in the aquatic plant *Myriophyllum aquaticum* and in hairy root cultures of *Catharanthus roseus* (Bhadra *et al*., 2001).

A serious problem with phytoremediation of TNT and RDX is that the contaminated soil and water at military firing ranges can contain concentrations of these chemicals that are phytotoxic. Obviously, only healthy and actively growing plants would be effective in taking up pollutants and fully metabolizing them. Although much research has been done to demonstrate the success of phytoremediation, resulting in its use on many contaminated sites, the method still lacks wide application. Its primary disadvantage when compared with engineering methods is that it is often considered too slow or only seasonally effective (Travis *et al.*, 2007).

For these reasons, attention has recently focused on ways to enhance the phytoremediation capacity of plants using either transgenic methods or endophytes (Hannink *et al*., 2002).

1.2.5 Rhizosphere degradation

Rhizodegradation, or enhanced rhizosphere degradation, takes place at the intersection of bioremediation and phytoremediation. In this process, the presence of the plant and its roots creates a rhizosphere zone more amenable to microbes that degrade contaminants. Root exudates such as organic acids and ketones may promote microbial growth such as the increase in soil organic matter caused by roots. Rhizosphere microbes have been reported to break down organic pollutants (Schnoor *et al*., 1995).

1.2.6 Rhizofiltration

Plants with large root systems usually absorb pollutants. Rhizofiltration is commonly used for treatment of industrial discharge, agricultural run off, metals and radioactive contamination. The movement of organic contaminants in soil depends on the chemical's relative water solubility, vapor pressure, and molecular size, charge and on the presence of other organics in the soil (Salt *et al*., 1997).

1.2.7 Phytostabilization

In phytostabilization, the contaminant is not destroyed, but simply prevented from migrating offsite or doing further damage to the ecosystem. A disadvantage of this technique is that ensuring that contaminant migration does not occur may involve monitoring a site for the seeable future. Most of the organic chemical contaminants are lypophilic and are attracted to the hydrophobic surfaces on organic matter, such as humus and plant cell wall components or soil particles (Cunningham *et al*., 1996; Noctor *et al*., 1996). In

this aspect, plants are used only to reduce the bioavailability of environmental pollutants (Salt *et al*., 1997).

1.2.8 Phytovolatization

In volatilization, the plant takes up the contaminant of concern from soil, water, or a mixture of both, converts it to a volatile form, and releases it to the atmosphere, usually through the leaf stomata (Burken and Schnoor, 1998). This technique is only suitable for contaminants that do not pose a significant air pollution hazard. Contaminants taken up by the roots pass through the roots to the leaves and are volatized through stomata where gas exchange occurs (Nedelkoska and Doran, 2000).

1.2.9 Organic pumps

Trees with dense roots take up large volumes of water such as poplars and cottonwoods, which decrease the tendency of surface pollutants to move downward towards ground water and into drinking water, generally used to control agricultural run off and landfill leaching.

1.2.9.1 Cell cultures

Plant cell cultures have been used for investigating the phytotoxicity and metabolic fate of the xenobiotics (Harms, 1996). Results are compared with those of the intact plants grown under normal conditions. Qualitatively, the metabolites of degradation are the same in both systems. But the advantage of cell suspension cultures over intact plants is that the results may be obtained more quickly with less expense of analytical techniques. Most of the

phytoremediation studies are carried out using whole plants grown either in soil or hydroponically.

In vitro culture of plant organs such as roots and shoots allows indefinite propagation and experimentation using tissues derived from the same plant, avoiding the risks of variability among species. Goel *et al.* (1997) have shown the degradation of xenobiotic nitrate ester, nitroglycerin by *Beta vulgaris* cell cultures and cell extracts. Glycerol trinitrate (GTN) was degraded to glycerol mononitrate (GMN) *via* glycerol dinitrate (GDN). Bioreactor studies were also conducted to evaluate the process of phytoremediation in large scale. Knops *et al.* (1995) have used a 3 liter airlift bioreactor for studies on degradation of 4-nitrophenol using soybean cell suspension cultures.

Czuba (1987) reported that in callus of *Daucus carota* and *Lactuca sativa*, the methyl mercury toxicity is partly a hormonemediated by the auxin 2, 4–D and light-sensitive event. PCBs known for their toxicity, carcinogenicity, wide distribution and slow biodegradation were also effectively degraded into their metabolites by plants, sterile cultures of *Solanum nigrum*. Campanella (2000).

1.2.9.2 Hairy roots

Hairy root cultures, known for their fast growth, biochemical stability and hormonal autotrophy, have been tested successfully for various biotransformation studies and phytoremediation, particularly hyperaccumulation of heavy metals (Hughes *et al*., 1997). Extensive root proliferation caused by *Agrobacterium rhizogenes*, generally considered an undesirable characteristic, may find good utility for phytoremediation as roots

retrieve the contaminant from deeper soils due to their larger penetrating ability, and also the enzymatic degradation (peroxidases, laccases and oxygenases). Paul and Campanella (2000) have shown the biodegradation of anthracene using *Medicago sativa* hairy root cultures. *Catharanthus roseus* hairy root cultures were reported to degrade the explosive TNT successfully (Hughes *et al.,* 1997).

Hairy roots of *Solanum nigrum, Nicotiana tabacum, Thlaspi caerulescens* removed Cd^{+2} efficiently (Nedelkoska and Doran, 2000). Wu and Cenderbaim, (2001) have reported hyperaccumulation of Cd^{+2} by hairy root cultures of *Adenophora lobophylla*, which is an endangered species reported to have high phytochelatin content. Phytoremediation aspects are illustrated in Figure 1.2 in which Salt *et al.* (1997) described various phytoremediation treatments.

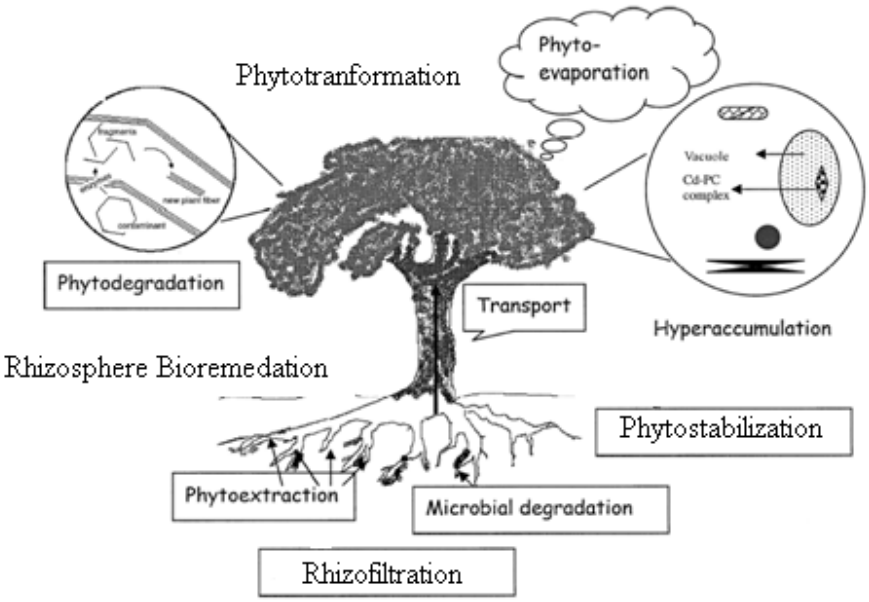

Figure (1.2): Steps involved in phytoremediation with modification (Doty, 2008).

1.2.9.3 Enzymology

The green liver concept proposed by Sandermann (1992) describes the function of plants as global sink for environmental pollution by xenobiotic metabolism. The similarity of plant enzymes with those of liver in action on xenobiotics by 3 phases, transformation, conjugation and compartmentation, describes the fate of xenobiotic metabolism in plants. Various plant xenobiotic enzymes, *viz*. cytochrome P450, glutathine–S-transferases, carboxyesterases, *O*-glucosyl transferases, *O*-malonyl transferases, N-glucosyl transferases and N-malonyl transferases, were isolated, purified and proved effective against various xenobiotics such as PCBs, organic solvents and chlorinated pesticides. Interestingly, the molecular masses were similar in some plants, to those of liver.

Sandermann (1994) described various plant glutathione-S transferases acting on xenobiotics from 59 different plant species and four plant cell suspension cultures and some species were found to have high activities.

Many of the plant xenobiotic enzymes are constitutive, unlike microbial enzymes, which are inductive, requiring prior induction for action, since extensive metabolism of numerous xenobiotics was observed in plant cell cultures without any prior induction treatment (Sandermann, 1992). Thus, plants as tools for remediation of contaminants have gained importance.

Extra cellular enzymes released by plants degrade xenobiotics in soils, including laccases, dehalogenases, nitroreductases, nitrilases and peroxidases, were proved to be effective against a number of contaminants ranging from

organic solvents to explosives like TNT (Schnoor *et al*., 1995; Boyajian and Carreira, 1997). Even though the enzymes are constitutive, some enzymes (enzymes for alachlor-[2-chloro-2-6-diethyl-N-(methoxymethyl)-acetanilide] were reported to be induced, such as glutathione transferases by herbicide antidotes (Carreira and Wofy, 1992). Schnoor *et al.* (1995) isolated an enzyme from residue that had TNT degradation potential, which was of a plant but not bacterial origin. Glutathione-S-transferase enzymes play an important role in xenobiotic metabolism and antioxidative protection and are well characterized in bacteria, plants as well as in mammals (Pflugmacher and Sandermann, 1992). They also play a role in the auxin response and in plant secondary compounds metabolism such as anthocyanin and cinnamic acid (Marrs, 1996).

During transformation process (Phase I), the enzymes oxidize, reduce or hydrolyze the substrate introducing a reactive group such as glutathione or glucuronate catalyzed by phase II enzymes (Sandermann, 1992).

Soluble GSTs form dimers, each subunit of which contains active sites that bind glutathione and hydrophobic ligands (Edwards *et al*., 2000). During the conjugation process, the ATP-binding cassette (ABC) transporters, which are also called glutathione-S-conjugates in the roots, recognize oxidized diglutathione (GS-SG) glutathione conjugates of organics (Pflugmacher and Sandermann, 1992; Lu *et al*., 1998).

The ABC transporters contain a cytoplasmic domain (the ABC protein) that binds and hydrolyzes ATP to strengthen solute translocation across the cytoplasmic membrane (Grangeiro *et al*., 2004). Further processing of xenobiotics [O-*ß*-D-glucosides] starts with addition of an O-malonyl

subconstituent, this acts as a signal for transport into the vacuole (Grotz *et al*., 1998) or into the extracellular space for subsequent degradation (Chaney, 1988; Baker, 1999). The conjugates are rapidly metabolized in the vacuole to cysteine conjugates by peptidases action, which further get metabolized in the cytosol to other products and excreted as bound residues in the apoplast, exudates in the rhizosphere or volatiles from aerial tissues (Coleman, 1992).

Nitroreductases action of certain plant species on nitroaromatic compounds like TNT, hexahydro-1,3,5-trinitro- 1,3,5-triazine (RDX), and GTN was shown by various researchers leading to the final products such as CO_2 and ammonium or nitrate (Travis *et al.,* 2007).

1.2.9.4 Cytochrome P450-containig monooxygenases

Cytochrome P450-containig monooxygenases (EC 1.14.14.1) belongs to one of the major classes of enzymes that are responsible for detoxification of organic and inorganic contaminants in animals and plants (Robineau *et al*., 1998). They are mixed-function oxidases located in the membranes of the endoplasmic reticulum (microsomes), that utilize NADPH and/or NADH reductive equivalents for the activation of molecular oxygen and for the incorporation of one of its atoms into hydrophylic organic compounds (XH) that produce functionalized products (XOH) (Schuler, 1996). In this case the second atom of oxygen is used for the formation of a water molecule. The microsomal cytochrome P450 containing monooxygenase system is the electron transfer chain, located in the membranes of the endoplasmic reticulum. This system contains the following components: the initial stage of electron transfer is an NADPH-cytochrome P450 reductase (EC 1.6.2.4); the

intermediate carrier, cytochrome b5; and the terminal acceptor of electrons, cytochrome P450. When NADPH is used as the only source of reductive equivalents in this system, the existence of an additional carrier, an NADH-dependent flavoprotein, becomes necessary. NADH may be oxidized also by the NADPH-dependent redox system (Figure 1.3). In the latter case b5 is not needed as the medium carrier (Hansikova *et al.,* 1994).

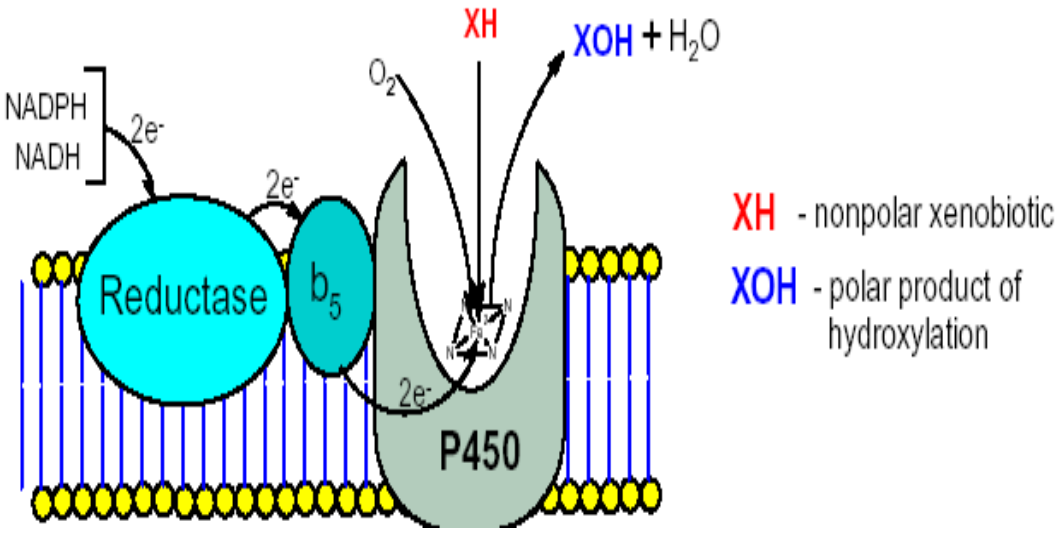

Figure (1.3): Microsomal monooxygenase system (Hansikova *et al.,* 1994).

In plants, Cytochrome P450 (or CYP) enzymes are involved in herbicides metabolism. P450, in cooperation with NADPH cytochrome P450 oxidoreductase (reductase), catalyzes oxidation reactions of lipophilic compounds, including certain herbicides (Figure 1.4). These P450 species play an important role in toxin selectivity and resistance (Coleman, 1992).

Cytochrome P450s catalyze extremely diverse and often complex region-specific and/or stereo-specific reactions in the biosynthesis or catabolism of plant bioactive molecules (Morant *et al*., 2003).

It is possible to list more than 20 physiologically significant processes and reactions, in which cytochrome P450 plays a key role (Durst, 1991; Schuler, 1996). The most important of them are: biosynthesis of lignine monomers (Whetten and Sederoff, 1995), anthocyanins (Holton and Cornish, 1995), furanocoumarins (Berenbaum and Zangerl, 1996), gibberellins (Jenings *et al*., 1993), isoflavonoid phytoalexins (Kochs and Grisebach, 1986), alkaloids, hydroxylation of fatty acids, limonene and geranoil (Kutchan, 1995).

At the same time some plant cytochrome P450 containing monooxygenases can play an important role in hydroxylation of exogenous toxic compounds (pesticides, environmental pollutants and other xenobiotics) after they penetrate into the plant cell (Sandermann, 1994). They also participate in the reactions of C- and N-hydroxylation of alyphatic and aromatic compounds, N-, O-, and S-dealkylation, sulpho-oxidation, deamination, N-oxidation, oxidative and reductive dehalogenation, etc. (Schuler, 1996). The biochemical resistance to many herbicides in plants is mediated by the rapid transformation of the herbicide into a hydroxylated, inactive product that is subsequently conjugated to carbohydrate moieties in the plant cell wall (Schuler, 1996). N-demethylation and ring-methyl hydroxylation of the phenylurea herbicide chlortoluron in wheat and maize are cytochrome P450-dependent processes (Fonné-Pfister *et al.,* 1990; Mougin *et al*., 1990).

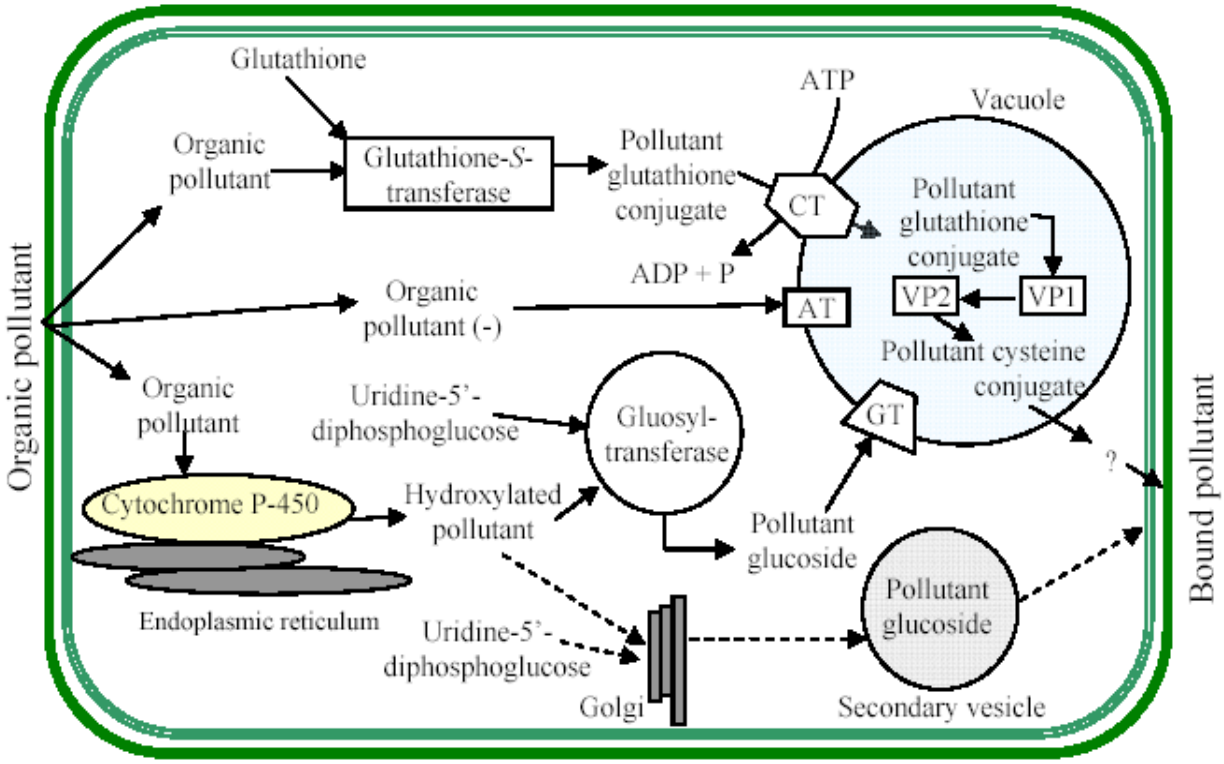

Figure (1.4): Description of plant metabolism of organic pollutants. Broken arrows represent proposed pathway for glucosylation in Golgi. CT is glutathione-conjugate transporter, AT is ATP-dependent anion transporter, GT is ATP-dependent glucosideconjugate transporter, and VP is vacuolar peptidase (Coleman, 1992).

1.2.10 Studied plants: *Sesbania grandiflora* and *Arabidopsis thaliana*

1.2.10.1 *Sesbania grandiflora*

Sesbania grandiflora L. Poir, common names in English are: Corkwood tree, Hummingbird tree, Scarlet wisteria tree, *Sesban, Sesbania*. In French: Sgati a grandes fleurs. Hawaiian: Ohai keʻkeʻo. Palauan: Katurai. Samoan:

Spain. Tahitian: Afai, Ofai, Ouai, Oufai (Heering and Gutteridge, 1992). The genus *Sesbania* is important in Africa and has a large number of species. Some of the most important members of this subgenus Agati are mainly found in southern Asia and its members are more of perennial and tree types as compared to the relatively more annual and shrub types found in the genus *Sesbania* (Onim and Dzowela, 2006).

1.2.10.1.1 Plant taxonomy

Domain: Eukaryota

Kingdom: Plantae- Plants

Subkingdom: Viridaeplantae

Phylum: Magnoliophyta

Subphylum: Euphyllophytina

Infraphylum: Radiatopses

Class: Magnoliopsida Dicotyledons

Subclass: Rosidae

Superorder: Fabanae

Order: Fabales

Family: Fabaceae Bean Family

Genus: Sesbania

Specific epithet: *grandifolia* - Poiret

Botanical name: - *Sesbania grandifolia* (Char, 1983)

Figure (1.5): *Sesbania grandiflora* L. plants at the early stage of growth.

1.2.10.1.2 Plant ecology

The plant is tolerant to a wide range of soils including alkaline soils that are poorly drained, saline, and low fertility. *S. grandiflora* shows some tolerance to acid soils with a pH down to 4.5 and it is well adapted to heavy clay soils (Heering and Gutteridge, 1992).

It is best adapted to a region with annual rainfall of 2,000-4,000 mm, but has been grown successfully in semi-arid areas with 800 mm annual rainfall and up to 9 months of dry season. The plant is tolerant to flooding over short periods. Its rapid early growth and erect habit usually enables *S. grandiflora* to access sunlight by overtopping neighboring plants. Birds pollinate the large hermaphroditic flowers and the plant is able to produce ripe pods 4-9 months after planting.

1.2.10.1.3 Plant uses

Several *Sesbania* species are sources of livestock feed, both as fodder and hay (Onim *et al*., 1985). The plant is valued as a fodder in many regions. In south-central Lombok, Indonesia, it grows around rice fields provide up to 70 percent of the diets of cattle and goats during the annual eight-month dry season. Plants are used to shade nurseries and some crops, and as an element of windbreaks. Leaves contain as much as 73% crude proteins in dry matter and have various uses in the herbal medical lore (Evans, 2001).

Dry matter yield of *S. grandiflora* forage is quite high when compared to other forage legumes like *Leucaena leucocephala*, *Pigeon pea* with an estimation of 8000, 5500, 3000 kilogram per hectare for *Sesbania*, *Leucaena* and *Pigeon pea* respectively after six months of planting (Onim, 1986).

Nitrogen yield of these legumes were 250, 175, and 120 Kg/ha for *Sesbania*, *Leucaena* and *Pigeon pea* respectively. The mean crude fiber content in *S. grandiflora* is low (13%) and the mean calcium to phosphorus ratio is high 3:8. It is therefore clear that *Sesbania* spp are forages of very high quality (Onim, 1986). *Sesbania spp* have been used for many years as a source of fuelwood. Bulk density of *Sesbania spp* varies according to species, rate of growth, and age. Values ranging 240-616 Kg/m^3 have been reported (Onim, 1986).

Several studies have shown that *Sesbania spp* returns into the soil as green manure between 80 and 120 kg of N within 90 days (Dargan *et al*., 1975). *Sesbania* spp are also often used in land reclamation especially in salty

(saline) and sodic soils as well as in mining and excavation sites (Srivastava *et al*., 1973; Malik and Haider, 1977; Yousir, 2008).

1.2.10.2 *Arabidopsis thaliana*, the weed that becomes a model plant

Kingdom	Plantae – Plants
Subkingdom	Tracheobionta – Vascular plants
Superdivision	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Subclass	Dilleniidae
Order	Capparales
Family	Brassicaceae – Mustard family
Genus	*Arabidopsis* Heynh. – rockcress
Species	*Arabidopsis thaliana* (L.) Heynh. – mouseear cress

Arabidopsis thaliana is a small flowering plant that grows in mild climate all over the world and has numerous features ideal for laboratory research making it the organism of choice in plant biology (Figure 1.6). *Arabidopsis* is a member of the mustard (Brassicaceae) family like radish and cabbage, and was first discovered by Johannes Thal (hence, *thaliana*) in the Harz Mountains in the sixteenth century and initially classified as *Pilosella siliquosa*. Currently, over 300 wild accessions are now available from the *Arabidopsis* stock centres in Ohio, USA and Nottingham, UK providing a valuable resource for genetic analysis of natural variation (Kunkel, 1996).

Figure (1.6): *Arabidopsis thaliana* plant after 30 days from seed sowing (10 X).

The potential of *A. thaliana* as a model organism for genetics was first recognized in 1943 by F. Laibach (Al-Daoude, 2003). Although *Arabidopsis* has no major agronomic significance, it offers several advantages for researchers in many areas of plant biology especially in molecular genetics (Wilson, 2000). Recognized as a tool for the discovery of genes in the role of transformation and phytoremediation, *Arabidopsis* has been used for phytotransformation studies. One such study examined the TNT metabolic pathways within *Arabidopsis* (Travis *et al*., 2007). Heavy metal uptake using *Arabidopsis* genes has been more extensively studied such as for arsenic (Rivera *et al.,* 1998) and the elements Zn, Co, Cu, Pb and Mn (Parameswaran *et al*., 2007). The use of this model plant in molecular studies has several advantages.

Sequenced plant genome (~125Mbp, containing about 23000 genes) is publicly available. The plant has a rapid life cycle about six weeks from seed

to seed; productive seed production and easy cultivation in restricted space; an individual self-fertilized plant can produce thousands of seeds compared with out-crossing plants (Abbot and Gomes, 1989; Meyerowitz, 1989). It can be efficiently transformed by a single *Agrobacterium tumefaciens* dip method. A large number of mutant lines and genomic resources are available from different seed stock canters such as the *Arabidopsis* Biological Resource Center (Al-Daoude, 2003). Insertional mutagenesis has been used to create "gene knockout" at the University of Wisconsin-Madison (Sussman *et al*., 2000).

1.2.11 Phytoremediation with transgenics

A direct method for enhancing the effectiveness of phytoremediation is to overexpress in transgenic plants the genes involved in metabolism, uptake, or transport of specific pollutants (Rugh, 2004; Cherian and Oliveira, 2005). The introduction of these genes can be readily achieved for many plant species using *Agrobacterium tumefaciens*-mediated plant transformation. As phytoremediation is generally more effective when using large, fast-growing plants, and willow transformation protocols have not yet been published, the focus has been on poplar. Depending on the hybrid and particular clone, reasonable transformation frequencies can be achieved in poplar trees (Han *et al*., 2000).

1.2.11.1 Transgenic phytoremediation of organic pollutants

Phytoremediation of nitroaromatics was significantly improved with transgenic plants (Rosser *et al*., 2001). As nitroaromatic explosives are

phytotoxic, phytoremediation of these pollutants using nontransgenic plants is severely hindered. However, when bacterial genes involved in nitroaromatic degradation were expressed in plants, the plants became more tolerant to the pollutant and could more readily remove it.

Increased removal rates of a variety of small organic compounds were achieved by overexpressing cytochrome P450 gene a class of enzymes involved in the metabolism of xenobiotics in mammals, plants, and bacteria. The common pollutants TCE, carbon tetrachloride, chloroform, benzene, and vinyl chloride are all substrates of the mammalian isoform P450 IIE1, which is encoded by *CYP2E1*. When the *hCYP2E1* was overexpressed in tobacco plants, the transgenics produced hundreds of times more TCE metabolite than did the nontransgenics, and removed 98% of the ethylene dibromide, another substrate of the P450 2E1 enzyme, compared with 63% removal by the null vector control plants (Doty *et al*., 2000). The P450 2E1 enzyme from rabbit was successfully expressed in hairy root cultures of *Atropa belladonna* (Banerjee *et al*., 2002). These mammalian enzymes functioned well in plants without any need to modify the gene or to include the other enzymes, oxidoreductase and cytochrome b5, known to be required for full function of mammalian P450s. Apparently the plant versions of these common enzymes are sufficiently similar to the mammalian ones that the P450s can function with either type. In another study, the *CYP2E1* gene was also overexpressed in hybrid poplar (*Populus tremula* × *Populus alba*) (Doty *et al.,* 2005).

TCE metabolism in transgenic poplar removed volatile TCE and benzene from air at greater rates than did the control plants. While the nontransgenic

poplar remove a significant amount of TCE from air, the *CYP2E1* transgenic plants removed nearly 80% of the TCE in 1 week.

Furthermore, expression of mammalian cytochrome P450 genes in transgenic plants has also been used to detoxify herbicides. CYP1A1, CYP2B6, and CYP2C19 enzymes metabolize a wide range of herbicides (Inui *et al*., 2001). Rice (*Oryza sativa*) plants expressing enzymes that degrade herbicides may be helpful in reducing the load of herbicides in paddy fields and streams (Hirose *et al*., 2005). Transgenic rice with *CYP2B6* germinated well on medium containing 25 ppm alachlor or 50 ppm metolachlor and on medium with herbicides while nontransgenic rice did not grow at all (Doty *et al*., 2007).

Transgenic *Arabidopsis* that expressed the aromatic-cleaving extradiol dioxygenase (DbfB), and transgenic tobacco plants that expressed haloalkane dehalogenase (DhaA) were constructed to produce cytoplasmic or secrete forms of the enzymes. The transgenic plants producing enzymes were more tolerant to higher concentrations of the pollutant, and more of the dehalogenated product was found in the hydroponic medium. When exposed to crystals of 2,3-dihydroxybiphenyl, only the transgenic *Arabidopsis* expressing the apoplast-targeted DbfB induced ring cleavage of the pollutant. These studies demonstrate the usefulness of engineering plants with secreted enzymes for pollutants that are either too phytotoxic or less bioavailable (Al-Daoude, 2003).

1.2.11.2 Transgenic phytoremediation of metals

Since metals cannot be metabolized or broken down to less toxic forms, the goal of remediating was metal-contaminated soil is generally to extract the metal from the large soil volume and transfer it to a smaller volume of plant tissue for harvest and disposal. For metals such as mercury and selenium, an alternative strategy is to convert the metal to a volatile form for release and dilution into the atmosphere. Phytoremediation of a metal requires high-biomass plants that can tolerate the metal, translocate it from the roots to the shoots, and compartmentalize the metal or modify it for volatilization (Kramer and Chardonnens, 2001; Pilon-Smits and Pilon, 2002; Rugh, 2004; Eapen and D'Souza, 2005; Meagher and Heaton, 2005).

Many of the genes involved in metal uptake, translocation, and sequestration have been identified using the model plant *Arabidopsis* or naturally hyperaccumulating plants. However, the phytoremediation capacity of these natural hyperaccumulators is limited by their small size, slow growth rates, and limited growth habitat. Therefore, if the genes are transferred to plant species such as poplar and willow with their high biomass and extensive root systems, significant removal of the heavy metals should be achieved (Meagher and Heaton, 2005).

Dhankher *et al.* (2002) constructed *Arabidopsis* plants with γ-*ECS* gene and the arsenate reductase C (*ArsC*) gene to control both the mobility and the sequestration of arsenic. Transgenic plants co-expressing these two genes grew substantially better, with healthy shoots, on a medium containing 200 μM arsenate compared with the wild-type controls.

By expressing organomercurial lyase (*merB*) within the same plant, the full pathway from methyl mercury to the least toxic metallic mercury was accomplished. *Arabidopsis* plants transformed with this gene were tolerant to concentrations of methyl mercury 50 times higher than the concentrations to which wild-type plants were tolerant and 10 times higher than those to which plants transformed with *merB* alone were tolerant(Bizily *et al*., 2000).

Other strategies are to overexpress ATP sulfurylase (Pilon *et al*., 2003), glutathione synthetase (Liang *et al*., 1999), or γ-*ECS* (Zhu *et al*., 2002) in Indian mustard (*Brassica juncea*). In a reported field trial on transgenic plants for enhanced remediation, three transgenic lines showed increased accumulation of selenium in leaves. Transgenic Indian mustard plants overexpressing the adenosine triphosphate sulfurylase (*APS*) gene accumulated 4.3-fold more selenium in the leaves than the wild-type plants. Transgenics overexpressing the γ-*ECS* gene or the *GS* gene accumulated 2.8- fold and 2.3-fold more selenium in their leaves than wild type respectively. The plants accumulated cadmium, lead, copper, zinc, nickel, and boron (Banuelos *et al*., 2005).

Under hydroponic conditions, the transgenics accumulated 24-fold more cadmium in roots and 3-fold more in foliage, and 36-fold more lead in roots and 9-fold more in foliage, compared with wild-type plants. In leaves of the transgenic plants, 12-fold more copper was accumulated than in wild-type plants. The transgenic tobacco plants grew better than nontransgenic control plants in all mining soils tested, and had much more biomass than the natural hyperaccumulator *Thlaspi caerulescens*. In another study, tolerance to lead and

cadmium was increased when a yeast ABC transporter family member, YCF1, was expressed in *Arabidopsis* plants (Song *et al*., 2003).

Therefore, the ideal plants would need to have enhanced capabilities of remediating both classes of pollutants. Using genetic and biochemical methods, it should be possible to clone the genes involved in remediation of both types of pollutants, and combine them in transgenic plants. It has been demonstrated that multiple genes can be transferred to plants using *Agrobacterium* by co-infecting with strains containing different constructs (Li *et al*., 2003; Doty, 2008). As a greater understanding of the genomics behind the ability of some organisms to modify or remove pollutants is gained, the potential to make phytoremediation a viable alternative to engineering solutions to environmental pollution has increased.

1.2.11.3 Phytoremediation of radionuclides

During in the 20th century, nuclear technology was initiated by the onset of the Cold War (Negri and Hinchman, 2000) where, nuclear bombs and nuclear reactor facilities were developed with radionuclides as the main constituent. Radionuclides are elements that can spontaneously produce high-energy radiation or light (Zumdahl, 1989). Therefore, radionuclides become a risk factor when exposed and/or deposited in soil and water. Sources of radionuclides were come from above-ground nuclear testing, accidents at nuclear reactor facilities, and fission by-products from nuclear bombs (Negri and Hinchman, 2000).

Radionuclides can also be deposited on the soil surface through mining, milling, and drilling for oil. Once radionuclides are deposited on the soil surface, they eventually are incorporated into the soil structure. Physical and biological nutrient cycles can distribute radionuclides throughout soil and water (Entry *et al*., 1997).

Natural weathering and chemical leaching can also release radionuclides into the soil (Dushenkov *et al*., 1999). Radionuclides can then absorb onto soil particles, bind with soil constituents, or remain in the soil solution. Soils with clay particles have a strong adsorption for radionuclides due to clay's large surface area. Soil constituents, such as organic matter and oxides, can temporarily bind radionuclides, allowing their release under specific environmental conditions. The amount of bound radionuclides is usually linearly correlated with time that has passed since the radionuclide deposition. When radionuclides are adsorbed or bound in the soil, then they are not bioavailable to plants, microorganisms, or soil invertebrates. However, when radionuclides remain in the soil solution, though, then they are bioavailable to soil biota (soil microorganism) and plants (Dushenkov, 2003).

Radionuclide bioavailability mostly depends on the type of radionuclide deposition, the time of deposition, and the soil characteristics (Dushenkov *et al*., 1997). Radionuclides ^{137}Cs and plutonium tightly bind to soil particles, decreasing their bioavailability. Four common radionuclides are ^{137}Cs, ^{90}Sr (strontium), ^{234,235,238}U (uranium), and $^{238\text{-}241}$Pu (plutonium). ^{137}Cesium and ^{90}Sr come from fission by-products while Pu is released from nuclear weapon testing and nuclear fuel facilities. Uranium is released from nuclear fuel

cycles, but is the only one of the four radionuclides that occurs naturally (Negri and Hinchman, 2000).

On the other hand, different phytoremediation mechanisms exist to treat radionuclide contamination depending on the type (metal or organic) and the location (water or soil) of the contaminant. One mechanism, called rhizofiltration, occurs when contaminants are in water sources, such as ponds, streams, or groundwater, and are adsorbed or precipitated onto or into plant roots (USEPA, 1998). Rhizofiltration is used mostly when radionuclide concentrations are too low for efficient removal by traditional methods and too high to be released into the environment (Dushenkov *et al*., 1997).

Two plants that have proven the most successful at rhizofiltration. They are sunflower (*Helianthus annuus*) and water hyacinth (*Eichornia crassippes*). Both have been found to accumulate significant percentages of radionuclides (^{137}Cs, ^{238}U, ^{90}Sr) within a few hrs to a few days. Biological indicator of radionuclides, *Amaranth* cultivars produce high above ground biomass, which accumulates high concentrations of ^{137}Cs up to 3000 Bq kg^{-1}. Maximum ^{137}Cs concentration reached after 35 days of growth (Dushenkov, 2003). Legume and *Umbelliferae* family species accumulate the highest level of ^{90}Sr.

Pine seedlings (*Pinus radiata*) and *P. ponderosa* accumulate 1.5-4.5% of ^{90}Sr in their shoots (Negri and Hinchman, 2000).

Chapter Two
Material
and
Methods

2 Materials and Methods

2.1 Materials

2.1.1 Apparatus and equipments

The following equipments and apparatus were used throughout the experimental work:

Apparatus	Company and origin
Autoclave	Raypa / Spain
Bench top centrifuge	Eppendorf / Germany
Compound Light Microscope	Olympus / Japan
Distillator	GFL / Germany
Electric balance	Mettler / Switzerland
Electric oven	Grant / England
Electrical shaker incubator	Grant / England
Gas Chromatography (GC)	Perkin–Elmer / England
Growth chambers	Raypa / Spain
Hot plate with magnetic stirrer	Gallenkamp / England
Laminar air flow cabinet	Telstar / Spain
Micropipette	Eppendorf / Germany
Millipore filter	Millipore and Whatman / England
PCR	Bio-Rad (USA)
pH-meter	Jenway / England
Spectrophotometer	Secoman / France
Vortex	Heidolph / Germany
Water bath	Grant / England

2.1.2 Chemicals

The following chemicals were used during this study:

Chemicals	Company and origin
Kinetin (KIN), $NaHPO_4$, NaH_2PO_4, ∞-Naphthaleneacetic Acid (NAA), Benzyl adenine (BA), Indole-3-acetic Acid (IAA), Indole-3-butryic Acid (IBA), Iodine, Kanamycin (Km), Carbencillin (Car), MS medium, LB medium, MG/L medium.	BDH-England
Agarose, Bromo Phenol Blue, dNTP Mix (dATP, dCTP, dGTP, dTTP) 10 mM each, EDTA, Ethanol absolute 99.5%, Ethidium bromide, Glucose, Isopropanol, Methanol, $MgCl_2$, NaOH, SDS, Tris-Cl, β-Mercaptoethanol, Tween 20, Silwet-77, MTBE.	Euroclone / Italy
Primers, PCR Buffer, 5X TBE (Tris- Borate - EDTA) Buffer, Taq DNA polymerase, NaOAc, Clorox, DNA ladder.	Damascus/ Syria.

All reagents and solvents were at least of analytical grade. Trichloroethalene (99%), Trichloroethanol (99%), Dichloroethene (99%), Chloral (99%), 1,1,1-Trichloro-2,2-bis (4-chlorophenyl) ethane (DDT 99%), 1,1,1-Trichloro-2,2-bis (*p*-chlorophenyl) ethylene (DDE 99%), 1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDD 99%), and Nitrate uranium oxide{UO_2 $(NO_3)_2$ 99%}were supplied by Sigma-Aldrich Company and brought by DHL Company, Damascus, Syria.

2.2 Methods

This work was carried out in the plant tissue culture Lab., Biotech. Dept., Al – Nahrain Univ. and in the plant tissue culture laboratories, Molecular and Biotechnology Department (Atomic Energy Commissions of Syria), during the period 16/11/2008– 1/11/2009.

2.2.1 Sterilization of equipments

All the glasswares were scrubbed with brush in a hot detergent bath. They were washed thoroughly with tap water, and then rinsed two to three times with distilled water. However, glasswares with discarded cultures, as well as contaminated ones, were autoclaved shortly in order to liquefy the agar and kill any contaminants that may be present. All the glasswares that needed scrupulously cleaning were boiled in a saturated solution of Sodium bicarbonate. After that, they were immersed in 30% nitric acid solution for about 45 minutes followed by thorough washing with tap water until all traces of acid were removed. Then they were washed in the usual way. The glasswares were further rinsed with distilled water and then dried in an oven at 200^{o}C for 2 hrs. Graduate cylinders, test tubes and flasks were plugged with absorbent cotton in order to avoid any contaminants to enter them. Petri dishes were wrapped with Aluminum foil and left in the autoclaved at 1.04 kg.cm^{2}, 121^{o}C, for 20 minutes for sterilization.

2.2.2 Plant material

Sesbania grandiflora seeds were obtained from the Biotechnology Dept., College of Science, Al-Nahrain University, Iraq. *Arabidopsis thaliana* seeds were obtained from the Atomic Energy Commissions of Syria.

2.2.3 Sterilization of explants

Seeds were sterilized according to an protocol by Rashied *et al.* (1996). Mature seeds were used as explants for callus induction. Seeds were placed in sterilized flasks and washed with sterile distilled water. Seeds were then soaked in the 70% v/v ethanol for one minute, followed by washing with sterilized distill water for one to two minutes. Seeds were further sterilized by continuous shaking with 50% v/v Sodium hypochlorite 5.25% (Clorox) for 20 minutes. The seeds were then placed on sterilized Petri plates having sterile filter papers with the help of forceps to remove excess water.

2.2.4 Preparation of plant tissue culture medium

Murashige and Skoog, 1962 medium (MS) was prepared as stock solutions by dissolving the compounds listed in table 2.1 in distilled water (750 ml), and then 100 mg/l Myoinositol, 30g sucrose were added. The pH was adjusted to 5.7-5.8 using HCl (1N), and then 8 g/l agar was added to solidify the medium, and completed to one liter using distilled water. The auxins IBA (0.0, 0.05, 0.1, 0.2 or 0.5) mg/l; NAA (0.0, 0.1, 0.2 or 0.5) mg/l; IAA (0.0, 0.05, 0.1 or 0.2) mg/l and the cytokinins BAP (0.0, 0.5 or 1.5) mg/l and KIN (0.0, 0.5 or 1.5) mg/l were added with different combinations as required. These components were placed on a hotplate magnetic stirrer till boiling, and then aliquots of 10 ml were poured into culture vessels. The culture media was autoclaved at a pressure of 1.04 kg. cm^2, 121°C, for 20 min, and then left at room temperature until use.

Table (2.1): MS medium components used as stock solutions for plant tissue cultures experiments.

Components	**Chemical formula**	**Weight (mg/l)**
Macronutrients		
Ammonium nitrate	NH_4NO_3	1650
Potassium nitrate	KNO_3	1900
Calcium chloride hydrate	$CaCl_2.2H_2O$	440
Magnesium sulphate heptahydrate	$MgSO_4.7H_2O$	370
Potassium phosphate monobasic	KH_2PO_4	170
Micronutrients		
Boric acid	H_3BO_3	6.20
Potassium iodide	KI	0.83
Manganese sulphate. tetrahydrate	$MnSO_4.H_2O$	16.9
Zinc sulphate. heptahydrate	$ZnSO_4.7H_2O$	8.60
Molybdic acid (sodium salt).$2H_2O$	$Na_2MoO_4.2H_2O$	0.25
Cupric sulphate pentahydrate	$CuSO_4.5H_2O$	0.025
Cobalt chloride hexahydrate	$CoCl_2.6H_2O$	0.025
Chelated Iron		
Sodium ethylene diamine tetraacetate	Na_2-EDTA	37.3
Ferrous sulfate heptahydrate	$FeSO_4.7H_2O$	27.8
Vitamins		
Thiamine.HCl (B1)	$Cl_2H_{17}ClN_4OS.$ HCl	0.1
Nicotinic acid (B3)	$C_8H_{11}NO_3$.HCl	0.5

Pyrodoxine.HCl (B6)	$C_6H_5NO_2$	0.5
Glycine(free base)	$C_2H_5NO_2$	2.0
Myoinositol	$C_6H_6(OH)_6$	100

2.2.5 Microorganism

A. tumefaciens strain harboring the CYP2EI construct was kindly provided by Dr. Sharon Lafferty Doty, Forest Resources College, University of Washington, U.S.A.

2.2.6 Plasmid and bacterium strain for transformation

Agrobacterium tumefaciens strain used in this study was C58Cl (Doty *et al.,* 1996) containing pSLD50-6 plasmid (Banerjee *et al.,* 2002) (Figure 2.1). pSLD50-6 was a binary vector containing rabbit *cyp2e1*(pollutant resistant), Kanamycin resistance gene (marker gene) in T-DNA region (Doty *et al.,* 2007).

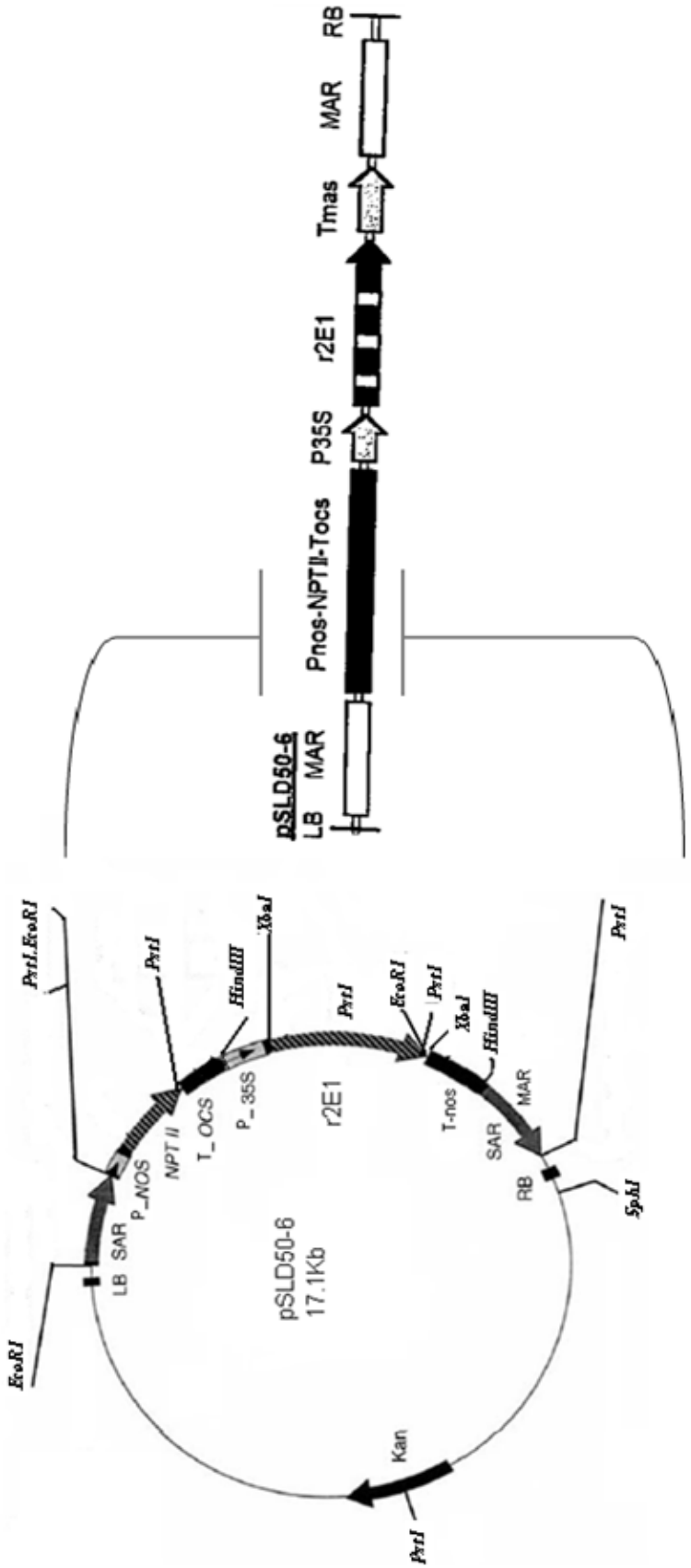

Figure (2.1) Plasmid map of pSLD50-6. LB, left border recognized by Agrobacterium VirD2; MAR, matrix attachment region; P-nos, nopaline synthase promoter; NPTII, neophosphotransferase; T-ocs, octopine transcriptional terminator; RB, right border recognized by Agrobacterium VirD2. (Doty *et al.*, 2007)

2.2.7 Bacteriological medium

Luria Bertani (LB) Broth (Miller, 1998) was used for growing *A. tumefaciens* stains whose components were listed in table 2.2, dissolved in distilled water (750 ml). Then 30g sucrose was added. The pH was adjusted to 7.2 using KOH (1N), and then 8 g/l agar was added to solidify the medium, and completed to one liter using distilled water. The medium was autoclaved at 1.04 kg. cm^2, 121°C, for 20 min, and then left at room temperature until use.

Table (2.2): LB medium components used as stock solutions for *A. tumefaciens* cultivation.

Component	Weight (mg/l)
Tryptone	10000
Yeast extracts	5000
NaCl	5000
Glucose	1000

The medium Garfinkel (MG) Broth (Huw *et al.,* 2005) was also used for growing *A. tumefaciens* stains whose components were listed in table 2.3, then 100 µl of stock solution was dissolved in distilled water (750 ml), and 30g sucrose was added. The pH was adjusted to 7.2 using KOH (1N), and then 8 g/l agar was added to solidify the medium, and completed to one liter using distilled water. The medium broth was autoclaved at a pressure of 1.04 kg. cm^2, 121°C, for 20 min, and then left at room temperature until use.

Table (2.3): MG medium components used as stock solutions for *A. tumefaciens* cultivation (Huw *et al.,* 2005).

Component	**Weight (mg/l)**
Mannitol	5000
L-Glutamic acid	1000
KH_2PO_4	250
NaCl	100
$MgSO_4 \cdot 7H_2O$	100
Tryptone	5000
Yeast extracts	2500
Biotin	1mg/ 100ml

2.2.8 Antibiotics (Doty *et al.,* 2000)

Antibiotics (Sigma) were used as selective agents. Antibiotics were prepared as stock solutions by dissolving the compounds listed in table 2.4. They were filter sterilized using 0.22 μm Millipore filter prior to use, and stored at 4°C then added post autoclaving.

Table (2.4): Stock solution of antibiotics.

Antibiotic	**Final concentration (μg/ml)**	**Solvent**
Carbencillin (Car)	100	Ethanol/Water
Kanamycin (Kan)	50	Water

2.2.9 Growth and conditions for *A. tumefaciens*

The strain of *A. tumefaciens* used in present study was C58Cl. It was grown in LB or MG/L medium supplemented with antibiotics such as Kanamycin (50 mg/l) and Carbencillin (100 mg/l). Cultures were grown at 28^{o}C for 16 hrs at 200-250 xg on a rotary shaker. Aliquots of *A. tumefaciens* cultures at hourly intervals were pipetted out and the optical density of the culture was measured at 600 nm (OD_{600} of 1-1.5) (Khan, 2003). Cultures showed a decline in the optical density after 18 hrs and thereby this time was used for all the experiments (Figure 2.2)

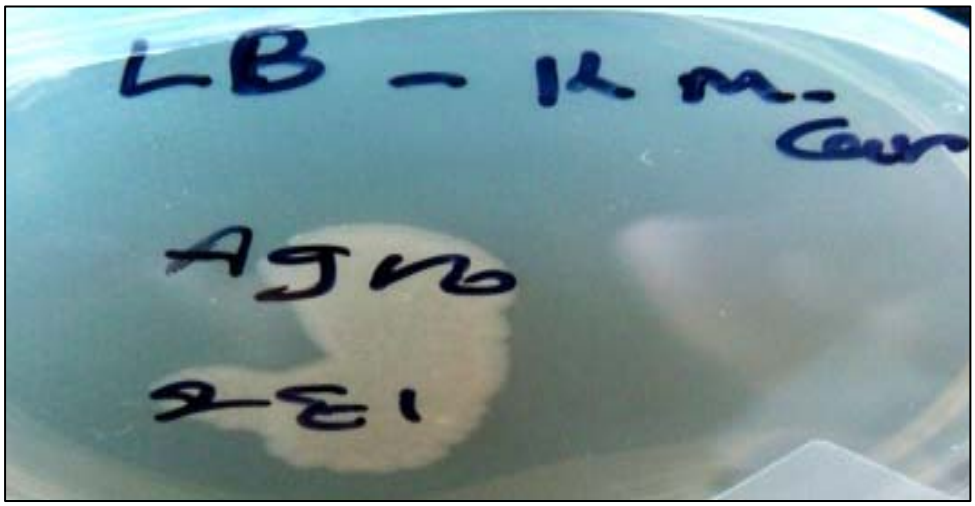

Figure (2.2): Overnight pure culture of *A. tumefaciens* C58Cl grown in LB medium at 28^{o}C and pH 5.7.

In *A. thaliana,* transgenic process was used by floral dipping with *A. tumefaciens*. Strain C58C1 (pSLD50-6) bearing the binary vector was grown in 500 ml of LB medium supplemented with the appropriate antibiotics at 28°C for 48 hrs using an orbital shaker (200-250 xg). Cells were harvested by centrifugation at 4000 xg for 10 minutes and pelleted cells were re-suspended

in 5% sucrose solution supplemented with Silwet-L77 (surfactant 0.02%).

In *S. grandiflora,* transgenic process was used for co-cultivation with *A. tumefaciens*. Semi-solid MS medium without antibiotics was supplemented with 100 mg/l Myoinositol, 30 g sucrose, 4 g agar were added. The pH was adjusted to 5.7-5.8 using HCl (1N). The culture media were autoclaved at 1.04 kg. cm^2, 121°C, for 20 min, and then left at room temperature until use. Overnight culture of *A. tumefaciens* having an OD_{600} of 1-1.5 was centrifuged at 4000 xg re-suspended in MS medium at different concentrations (1:10, 1:50, 1:75 or 1:100) and mixed using vortex. Co-cultivation of *S. grandiflora* cotyledonary explants with *A. tumefaciens* was conducted for different periods (30, 35 or 40 minutes).

2.2.10 Maintenance of isolate

2.2.10.1 Maintenance of bacterial isolate

Maintenance of bacterial isolate was performed according to Maniatis *et al.* (1982) and Atlas *et al*. (1995) as follows:

- Short- term storage

Colonies of bacteria were maintained for a period of 2-3 weeks on the surface of LB agar medium. The plates were tightly wrapped with parafilm and stored at 4°C.

- Medium - term storage

Bacterial isolates were maintained by re-culturing on slant LB agar medium for a period up to a few (2-3) months. Such medium was prepared in screw - capped vials containing 10-15 ml of the medium. The isolates were streaked on these slant media and incubated at 37°C for 24 hrs. Thereafter, the slants were taken and wrapped with parafilm and stored at 4°C.

- Long - term storage

Bacteria can be stored for one year in either nutrient broth or Brain-Heart infusion broth medium containing 15% glycerol at low temperature without significant loss of viability. This was done by adding 1.5 ml of sterilized glycerol to fresh preparation of bacterial growth in a small screw capped vials (Bejo bottles) with final volume of 10 ml and stored at -20°C.

2.2.11 Measurement of bacterial growth

Bacterial growth was monitored by Macfarlaned tube No. 5 turbidity standard which is equivalent to bacterial concentration for inoculums 1.5 x 10^8 cells / ml (Atlas *et al*., 1995)

2.2.12 Sterilization (Baily *et al*., 1999)

Two methods of sterilization were used as required:

- Moist heat sterilization

Media and solutions were sterilized by autoclaving at 1.04 kg.cm^2, 121ºC for 15 minutes

- Ultra filtration

Millipore filters (0.22µm) were used to sterilize sugars, amino acids and antibiotics.

2.2.13 Transformation

2.2.13.1 Seeds germination

Surface-sterilized *S. grandiflora* seeds were cultured directly on half strength liquid MS medium in a Petri dish with moistened sterile filter paper without any growth regulators. Also seeds germinated on half strength MS medium solidified with agar in flask (50 ml). Seed germination percentage was collected after 5 and 10 days (Figure 2.3).

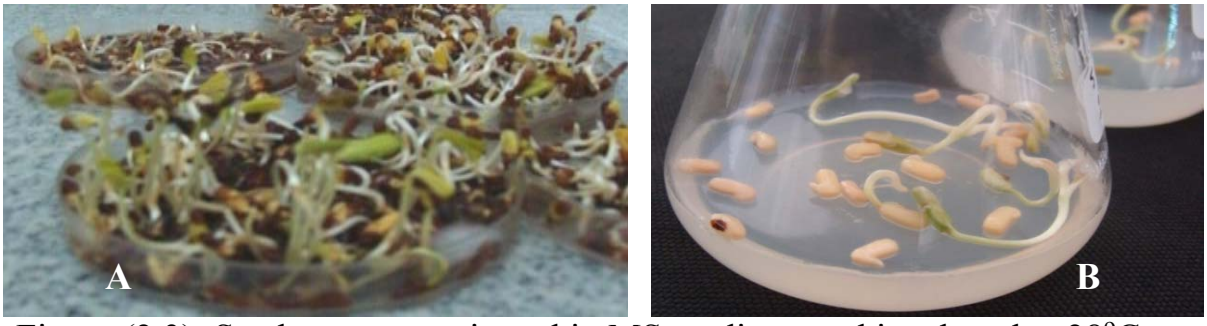

Figure (2.3): Seeds were germinated in MS medium, and incubated at 28°C.

A. Moistened sterile filter paper in Petri dish.

B. Seeds germinated on half strength MS medium solidified with agar in flask.

2.2.13.2 Determination of Kanamycin resistance (Kemal *et al.*, 1999)

S. grandiflora and *A. thaliana* seeds were germinated on MS medium containing different concentrations of Kanamycin (0, 50, 100 or 150 mg/l). Kanamycin resistance was carried out to determine the lethal dose of Kanamycin for both plants. MS medium was containing 5% sucrose and 8 g agar, pH of the medium was adjusted to 5.8. Results were observed after 10 days.

2.2.13.3 Co-cultivation of wounded explants with *A. tumefaciens* (Priya and Shivendra, 2009)

Cotyledonary explants were surface sterilized with 70% (v/v) ethanol for 10 seconds, then rinsed three times with Deionized sterile distilled water (DDH_2O), then 1% (v/v) of sodium hypochlorite solution (contains 5.25% Cl_2) was used for 10 minutes, rinsed three times with distilled water. All these processes were carried out inside the laminar air flow cabinate.

Cotyledonary explants were dissected from the 6-7 day-old *in vitro* seedling, transferred to a large glass Petri dish (14.4 cm in diameter) containing sterile water.

Cotyledonary explants were soaked in the co-cultivation solution for 30 – 40 minutes. After infection with *A. tumefaciens* all cotyledonary explants were blotted dry with sterile blotting paper to remove the excessive bacterial cells. Then explants were transferred to sterile MS medium. Petri dishes containing explants were sealed with parafilm and kept in dark at 25 ^{o}C inside incubator for 2-3 days (Figure 2.4).

Figure (2.4): *Sesbania grandiflora* transformation process.

A. Young cotyledonary seedlings.
B. Cotyledonary explants were dissected and wounded then co-cultivated with *A. tumefaciens.*
C. Cotyledonary explants were incubated in dark at 25^{o}C for 2-3 days.

After incubation for 2-3 days, the explants were washed 3-5 times with sterile water containing 50 mg/l Kanamycin and 400 mg/l Carbencillin to kill any *A. tumefaciens* cells still attached to the surface of explants. The inoculated explants were dried on sterilized filter paper, then transferred to pre-selective MS medium containing 50 mg/l Kanamycin and 400 mg/l Carbencillin. After 7- 10 days explants were sub-cultured on fresh selective medium to select transformants.

2.2.13.4 Plant regeneration (Thakur *et al.*, 2005)

Healthy calli were initiated and transferred to the regeneration medium consisted of MS medium supplemented with 100 mg/l Myoinositol, 30g sucrose, the auxins NAA (0.0, 0.1, 0.2 or 0.5) mg/l; IAA (0.0, 0.05, 0.1 or 0.2) mg/l and combination with cytokinins BAP (0.0, 0.5 or 1.5) mg/l and Kin (0.0, 0.5 or 1.5) mg/l were added. The pH was adjusted to 5.7-5.8 using HCl (1N). The culture media were autoclaved at 1.04 kg. cm^2, 121°C, for 20 min, and then left at room temperature until use. 50 mg/l Kanamycin as a selective agent and 400 mg/l Carbencillin were added after autoclaving. The regenerated shoots (Figure 2.5) were further transferred to MS medium containing auxins for root induction. Root induction medium was consisted of MS medium supplemented with 100 mg/l Myoinositol, 30g sucrose, IBA (0.0, 0.05, 0.1 or 0.2) mg/l and IAA (0.0, 0.05, 0.1 or 0.2) mg/l. The pH was adjusted to 5.7-5.8 using HCl (1N). The culture media were autoclaved as above then left at room temperature until use. A quantity of 50 mg/l Kanamycin as a selective agent was added after autoclaving.

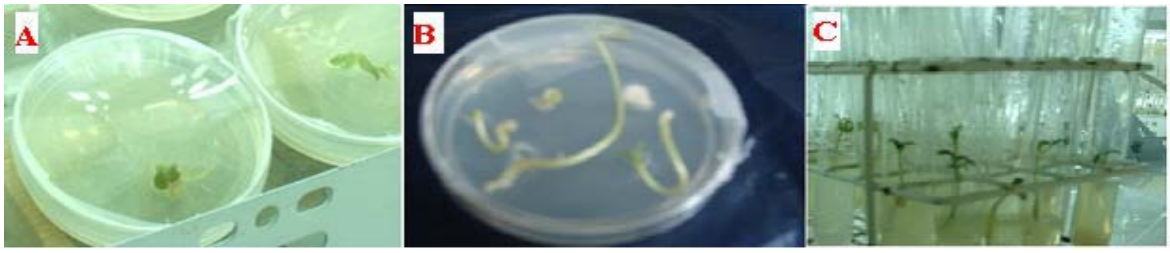

Figure (2.5): Shoot regeneration and root induction on MS medium containing 50 mg/l Kanamycin.

A. Callus induction

B. Shoot regeneration

C. Root formation

2.2.13.5 Establishment of plantlets in the glasshouse (Thakur *et al.*, 2005)

Well-rooted plantlets were washed with running tap water to remove agar from the surface of roots. They left inside test tubes containing tap water for a few days before transfer to pots in a glasshouse (Figure 2.6). Pots were containing soil: peat v/v at a ratio 1:1. Pots were transferred to a glasshouse with a relative humidity of 90%. All acclimatized plantlets survived the hardening process. Some plantlets were also transferred into 50 ml flasks containing half strength MS medium supplemented with Kanamycin 50 mg/l. They also displayed 100% survival.

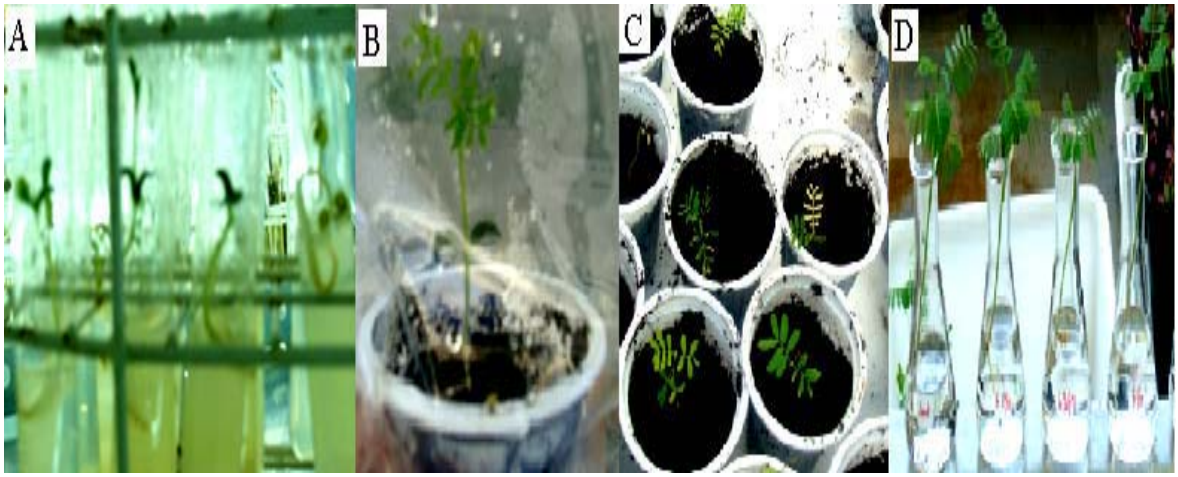

Figure (2.6): Plantlets were established in a glasshouse (A) and transferred to a 50:50 peat: soil mixture (B,C) or 50 ml flasks (D) containing half strength liquid MS medium supplemented with Kanamycin 50 mg/l.

2.2.13.6 Transformation of *A. thaliana* (Al-Daoude *et al.*, 2005)

A. thaliana plants were grown till flowering under long days in pots. Bolts were encouraged to proliferate to many secondary bolts after pinching. Plants with many immature flower clusters were used for transformation.

A. tumefaciens was resuspended to OD_{600} of 1-1.5 in 5% Sucrose solution then 100 ml for each two flower clusters were dipped. Silwet L-77 was added

in a concentration of 0.02 %. Flowers were dipped with *A. tumefaciens* solution for 2 to 3 seconds, with gentle agitation. Treated plants were covered with a dome for 16 to 24 hrs to maintain high humidity. Plants were left till seed maturity then collected and weighed. Seeds were surface sterilized as mentioned earlier then sown under sterile conditions using a 150 x 15 mm Petri dishes containing the Kanamycin (50 mg/l) incorporated with liquid MS medium. Petri dished were placed in an cooled incubator at 4°C for 2 days, thereafter transferred to light. Seedlings were then transplanted in pots containing 1:1 peat: soil (v: v).

DNA was isolated, purified and amplified using PCR. Plants showed successful transformation were further grown for 3-4 weeks for seeding. Seeds collected from regerants, and further experimentation (Figure 2.7). All contaminated glassware and plasticware, as well as soil, peat, etc, were sterilized after use at 120ºC, 4 hrs in a Pasteur oven, while greenhouse equipments were decontaminated with sodium hypochlorite.

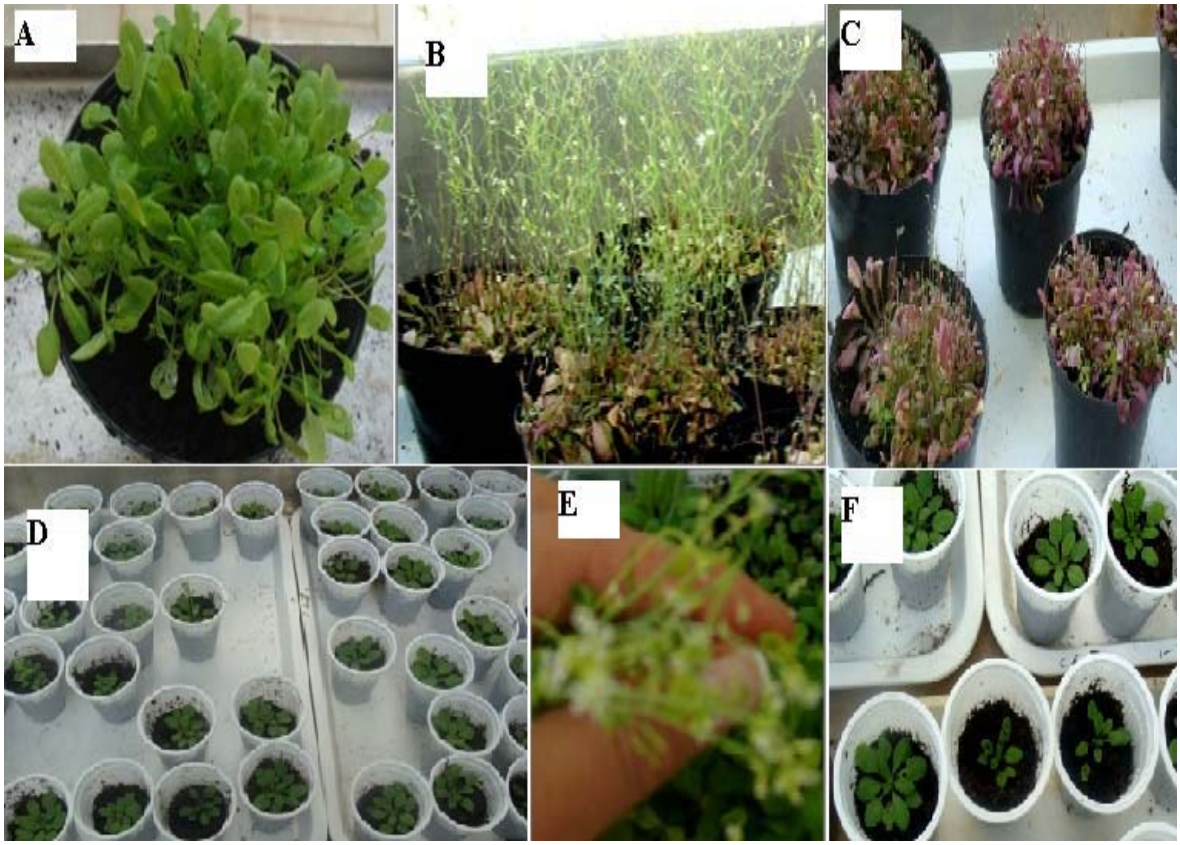

Figure (2.7): *A. thaliana* florets were dipped with aliquots containing *A. tumefaciens*.

A. *A. thaliana* grown in pots **B**. *A. thaliana* at full blooming **C.** *A. thaliana* after seed harvesting
D. Germination of *A. thaliana* F1 seeds **E.** *A. thaliana* F2 progeny flower harvest
F. *A. thaliana* F2 progeny grown for biochemical analysis

2.2.14 Isolation of bacterial DNA

2.2.14.1 Isolation of plasmid DNA

When plasmids with a high level of purity were required, Qiagen plasmid mini-prep and midi-prep kits were used according to the manufacture instructions. However, procedures routinely used for the isolation of plasmids from bacteria were based on the alkaline lysis method (Birnboim and Doly, 1979).

2.2.14.2 Reagents and chemicals

1. Solution I (2.25 ml Glucose 20%; 1.00 ml EDTA 0.5 M, pH 8.0; 1.25 ml Tris-HCl 1 M pH 8.0; 45.5 ml DDH_2O). This solution was autoclaved before use.
2. Solution II (0.4 ml NaOH 10 N; 1.0 ml SDS 20%; 18.6 ml DDH_2O). This solution does not require autoclaving.
3. Solution III (NaOAc 3 M, pH 4.8). 40.81 g of NaOAc was dissolved in a minimum volume of DDH_2O, pH adjusted to 4.8 with glacial acetic acid and the volume was made up to 100 ml with DDH_2O. This solution was autoclaved before use.
4. TE buffer (10 mM Tris-HCl and 1mM EDTA, pH 8) autoclaved before use.
5. Ribonuclease (RNase A) 10 mg/ml stock was made in 10 mM Tris-HCl pH 7.9 and 15 mM NaCl, heated to 100 ^{o}C for 15 min and allowed to cool slowly to room temperature. Finally, dispensed into aliquots and stored at -20 ^{o}C.

2.2.14.3 Protocol for plasmid DNA isolation

Aliquots of 10 ml of resuspended bacteria grown in LB medium were centrifuged at 6000 xg for 15 minutes at room temperature. Supernatant was discarded and 100 µl of solution I was added to the remaining pellet and vortexed. Tube contents were transferred to a fresh micro centrifuge tube, kept on ice for 10 minutes then 200 µl of solution II was added and mixed gently by inverting tubes several times and then left on ice for an additional 10 minutes solutions were spun for 10 minutes in an eppendorf centrifuge at full speed (10000-13000 xg) supernatant was transferred to another tube. Equal volume of isopropanol was added and mixed properly by inverting the tubes and then kept for 15 minutes at room temperature. Tubes were spanned for 10 min and supernatant was discarded. The pellet was washed with 70%

cold ethanol and dried. Pellet was resuspended in 50 µl of TE buffer. Finally DNA (3µl) was mixed with (1µl) of bromophenol blue dye. Samples were loaded carefully into the individual wells of an agarose gel, and then electrical power was turned on at 70 volt for 1 hr, afterwards the DNA migrated from cathode (-) to anode (+) poles. The Ethidium Bromide stained bands in the gel were visualized using UV transiluminator at 350 nm and photographed.

2.2.15 Isolation of plant DNA (Schaap *et al.,* 1989)

2.2.15.1 Reagents and chemicals

1. CTAB extraction buffer (1L stock): 730 ml of DH_2O, 100 ml of 1M Tris-HCl, pH 7.5, 140 ml of 5M NaCl, 20 ml 0.5M EDTA (pH 8.0), 10 g of Cetyltrimethylammonium bromide (CTAB) and 10 ml of 140 ml β-mercaptoethanol were mixed at warm temperature until completely dissolved. β-mercaptoethanol was added just prior to use.

2. TE buffer (100 ml stock): 1 ml of 1M Tris-HCl, pH 8.0, 0.2 ml of 0.5M EDTA (pH 8.0) and 98.8 ml of DH_2O were mixed together.
3. DNA precipitation solution (500 ml stock): 380 ml of absolute ethanol, 40 ml of 2.5 M sodium acetate (pH 6.3) and 80 ml DH_2O were mixed together.
4. DNA washing solution (100 ml stock): 76 ml of absolute ethanol, 1 ml of 1M ammonium acetate and 23 ml DH_2O were mixed together.
5. Chloroform/ isoamylalcohol solution (24:1)
6. Ice-cold isopropanol.
7. Pestle and mortar.
8. Ground glass.
9. Spectrophotometer (Gene Quant, GE Healthcare).

2.2.15.2 DNA isolation protocol

Genomic DNA was isolated from young leaves of control and transformed plants (*S. grandiflora* and *A. thaliana)* using standard protocol. The isolation of DNA was based on a many-step process using salting out methods: (Wu, 2001)

- Three hundred milligram of fresh leaves (50 mg of dry leaves) in liquid nitrogen.
- The material was transferred to 1.5 ml Eppendorf tube and 1 ml of wash buffer was added. The sample was vortexed for 5 minutes to remove polyphenols. Then spun at 12000 xg for 3 minutes
- The supernatant was removed and the washing step was repeated 4 to 5 times to take out sticky residues from the precipitant.
- CTAB (1000 µl) was added and centrifuged at 8400 xg for 5 minutes
- The supernatant was removed and 450 µl of resuspension buffer was added along with 80 µl of 10% SDS and incubated at 70ºC for 15 minutes
- The sample was allowed to cool to room temperature, then 300 µl of 7.5 M Ammonium acetate was added and placed on ice for 30 minutes then spun at 12000 xg for 15 minutes
- The upper clear aqueous layer was carefully transferred to another 1.5 ml Eppendorf tube.
- Equal amount of ice-cold isopropanol was added and centrifuged for 15 minutes at 12000 xg.
- The supernatant was discarded and the pellet was washed twice with 70% ethanol.
- The pellet was dissolved in 100 µl TE buffer.

- Ten microliters of RNase A (10 µg/ml) was added and incubated at 37ºC for 1 h.
- DNA was extracted with an equal volume of the mixture (chloroform: isoamylalcohol, 24:1), then spun at 12000 xg for 10 minutes
- The aqueous layer was transferred to a fresh 1.5 ml microfuge tube and 2 volumes of ice-cold ethanol were added, then spun at 12000 xg for 5 min at room temperature
- The pellet was washed with 70% ethanol.
- The pellet was dried in a speed vacuum and re-dissolved in 100 µl TE buffer or DDH_2O and stored at -20ºC until use.
- The DNA content was estimated by running on 2% agarose gel or by recording absorbance at 260 nm.
- For restriction digestion, 2 µg DNA was used, while 200 ng DNA was used for PCR amplification.

2.2.15.3 DNA visualization

A quantity of 2 µg DNA was mixed with 3 µl of bromophenol blue dye and loaded carefully into the individual wells of the gel, electrical power was turned on at 70 volt for 1 hr and the DNA moved from cathode (-) to anode (+) poles. Ethidium bromide stained bands were visualized using UV transiluminator at 350 nm and photographed.

2.2.16 Gel Electrophoresis (Maniatis *et al*., 1982)

After genomic DNA extraction, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA.

2.2.16.1 Reagents for Gel Electrophoresis

- Agarose
- 1 X TBE buffer
- Bromophenol Blue in 1% glycerol (loading buffer)
- Ethidium bromide
- DNA ladder

2.2.16.2 Protocol for Gel Electrophoresis

2.2.16.2.1 Preparation of an agarose gel

1. An amount of 1 X TBE (50 ml) was taken in a beaker, agarose powder (0.4 g) was added and the solution was heated until all gel particles dissolved.

2. Ethidium bromide (2 mg/ml) was added to the agarose solution and left to cool down at 50-60 ^{o}C.

2.2.16.2.2 Estimation of DNA concentration (Maniatis *et al*., 1982)

DNA samples that showed acceptable integrity were diluted to 1:10 or 1:20; afterwards the optical density was read with a spectrophotometer at a wavelength 260 nm. The concentration of DNA in µg/ml was calculated according to the following equation: (OD.260 nm x inverse dilution factor x 50 µg/ml = µg/ml).

For measuring the purity of DNA, readings were taken at wavelength 280 nm the purity of DNA was estimated = A 260 /A 280 = ~ 2

2.2.17 Primer design (Al-Daoude *et al.*, 2005)

Standard PCR primers ranged between 22- 23 bp long were designed using the Primer-Select program of DNASTAR. Primers were resuspended by dissolving in DDH_2O to a stock concentration of 100 μM, kept at –20°C and aliquots of 10μM were prepared as working solutions.

2.2.18 Polymerase chain reaction (Al-Daoude *et al.*, 2005)

Standard primers of 22-23bp long were designed using the primer-select program of the DNASTAR package following the method described by Hwei-Ming and Coon (1998). Primers were selected and PCR amplification was carried out to confirm the rabbit cytochrome p450 2E1 presence. Gene-specific PCR analysis was performed on 10 replicates of *A. tumefaciens* strain (C58Cl), 20 replicates of *S. grandiflora* and *A. thaliana* DNA prior to transformation.

Primers	Sequence	Length
Forward	5' CAT CGG GAA TCT TCT CCA GTT GG 3'	~0.4kb
Reverse	5' TGA AGG GTG TGC AGC CGA CAA 3'	

PCR was successfully amplified *CYP2E1* in all selected *A. tumefaciens*

2.2.18.1 PCR compounds

Optimization of a PCR reaction was accomplished after several trials; thus the following mixture was adopted.

Component	**Concentration**	**Amount (μl)**
Deionized water	---	12.7
PCR buffer	10 X	2.5
$MgCl_2$	50 mM	1.0

dNTPs	10 mM each	2.5
Primer A (forward)	10 picomols/ µl	2.0
Primer B (reverse)	10 picomols/ µl	2.0
DNA sample	0.05-0.15 µg/ µl	2.0
Taq DNA polymerase	5 unit/ µl	0.3
Total volume	---	25

2.2.18.2 PCR protocol

A. Molecular analysis of CYP2E1

For each plant (*A. thaliana* and *S. grandiflora*), a number of samples were tested according to phenotypes of the *CYP2E1* gene for both transformed and control plants. Two PCR reactions (two tubes) were performed for each sample: one for identifying the presence of *CYP2E1* and the other is the control (Doty *et al.,* 2003).

B. PCR Program

To detect the *CYP2E1* gene, the following program was adopted (Al-Daoude *et al.,* 2005).

Step	**Temperature (^{o}C)**	**Time (minutes)**	**No. of Cycles**
Initial denaturation	94	4	1
First loop			
Denaturation	94	1	30
Annealing	65	1	30
Extension	72	1.5	30
Final extension	72	5	1

2.2.18.3 Analysis of PCR products

PCR products and DNA ladder (200-2500 bp) were determined by electrophoresis. Aliquot of 3 µl of loading dye plus 10 µl of the product were mixed and loaded on 2% agarose gel (2 g agarose/ 100 ml 0.5 X TBE buffer) and run at 70 volt for one hr. The gel was stained with ethidium bromide solution (2 mg/ ml) for 15-30 minutes. Bands were visualized on a UV transsiluminator and then photographed using photo documentation system (Bio-Rad, USA).

2.2.19 Western blot analysis (Doty *et al.*, 2007)

2.2.19.1 Reagents of Western blot analysis:

- SDS 1.53 g (Resolving in 100 ml)
- Defatted milk 5%
- Triton X-100
- Tris-buffered saline (TBS) 20 mM
- NaCl 0.5%
- Tween 20 0.5%
- X-ray film (NIF RX, Fuji Photo Film Co. Ltd.).

2.2.19.2 Protocol of Western blot analysis for protein extraction

Total root protein was extracted by mixing powdered roots with SDS loading buffer (2 ml/g of fresh weight), boiled for 5 min, and centrifuged at 12000 xg for 5 minutes; SDS-PAGE gels were run with a Bio-Rad miniProtein II, and proteins were transferred to poly (vinylidene difluoride) membranes with Amersham Pharmacia Mighty-Small Transfer Unit at 400 mA for 1 hr. After blocking for 1 hr with 5% defatted milk in Tris-buffered

saline (TBS), blots were incubated with diluted rabbit anti-human P450 2E1 that was kindly provided by K. E. Thummel (University of Washington, Gentest, Woburn, MA for 1 hr. Blots were washed three times with TBS buffer and Tween 20 (0.1%) then incubated with the appropriate peroxidase-conjugated secondary antibody. Blots were washed four times with wash buffer (20 mM Tris, pH7.4/0.5 M NaCl/ 0.5% Tween 20). Blots were developed using Amersham Pharmacia's Enhanced ilumi according to the manufacturer's instructions.

2.2.20 Effect of pollutant compounds

2.2.20.1 Effect of Dichlorodiphenyltrichloroethane (DDT)

DDT stock solution (99% Sigma) was prepared at three concentrations (0, 10 or 20 μg/ml). Standard solution with different concentrations of DDT (0, 10 or 20 μg/ml) was used in these experiments after mixing with half strength MS medium (without agar). Experiments were performed with two plant species (*A. thaliana* and *S. grandiflora*).

2.2.20.1.1 Germination in different concentrations of DDT

A. thaliana and *S. grandiflora* seeds were germinated separately on MS liquid medium containing different concentrations of DDT (0, 10 or 20 μg/ml) with three replicates for each concentration and each replicate included 100 seeds from each pant. Seeds started germinating after 7 days and germination percentages were recorded after 15 days. All treatments were placed under sterilize greenhouse conditions, 25°C for 16/8 hrs. light/dark photoperiod using day light inflorescent at light intensity of 3000 lux.

2.2.20.1.2 Transgenic experiments in the presence of DDT

Sesbania transgenic and F2 *Arabidopsis* plants were cultured in a glass flask (50 ml) containing MS liquid medium supplemented with DDT (10 µl/ ml) with three replicates. Wild-type plants of each plant species (*Sesbania* and *Arabidopsis*) were similarly treated. All treatments were placed on a slow rotary shaker under sterile greenhouse conditions, 25°C for 16/8 hrs light/dark photoperiod using day light inflorescents at a light intensity of 3000 lux for 12 days. Plant samples were prepared for analysis by Gas Chromatography (GC), by cleaning and drying at 105^{o}C to a constant weight and homogenized. The obtained powder (0.3 g for each sample) was pressed into an Eppendorf tube and the samples were transferred to GC section for analysis.

2.2.20.1.3 Extraction of DDT from plant tissues (Chiu *et al.,* 2008)

A quantity of 0.5 g tissue was dried in an electric oven at 70^{o}C for 24 hrs. Plant tissue and media were extracted by hydrodistillation for 3 hrs using a cellulose thimble and insulted into Sohxlet flask containing 120 ml of Hexane: acetone mixture (1:1,V:V). Extraction using Sohxlet was carried out at 60^{o}C for 3.5 hrs. Extract was transferred into a conical bottom tube; Sohxlet and flask were washed three times with the extraction solution (2ml). Extract was then reduced in volume using stream of nitrogen khaldal (Turbo Vap LV) then DDT was stored under -10C^{0} for GC analysis.

2.2.20.1.4 Calibration curve of DDT

DDT solutions were prepared at five concentrations (0, 10, 15, 35 and 65 µg/ml). The true value was calculated by using rectum line equation (Y= aX + b) which gave sigenificant R^2 (Figure 2.8).

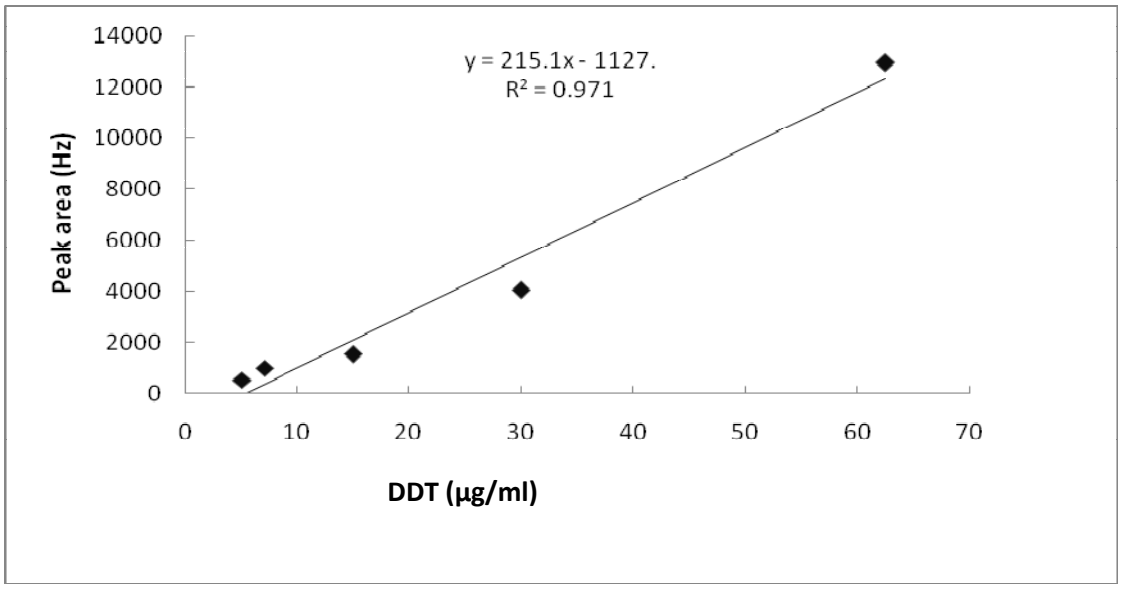

Figure (2.8): Calibration curve of DDT. R^2 value is 0.971.

2.2.20.2 Effect of Trichloroethane

TCE stock solution (99% Sigma) was prepared at three concentrations (0, 2, and 4%), 2% was prepared by dissolving 2ml of TCE stock solution (99%) in 98 ml sterilized distilled water and 4% was prepared by dissolving 4ml of TCE stock solution (99%) in 96 ml of sterilized distilled water.

Standard solution with different concentrations of TCE (0, 2, and 4%) was used in these experiments after mixing with half strength MS medium (without agar). Experiments were performed with two plant species (*A. thaliana* and *S. grandiflora*).

2.2.20.2.1 Germination in different concentrations of TCE

Seeds of both plants were germinated separately on MS liquid medium containing different concentrations of TCE (0, 2, or 4%). with three replicates for each concentration and each replicate included 100 seeds for each plant. Seeds started germination after 7 days and germination percentages were

recorded after 15 days. All treatments were placed under sterilized greenhouse conditions at 25°C for 16/8 hrs. light/dark photoperiod using day light inflorescents at a light intensity of 3000 lux.

2.2.20.2.2 Transgenic experiments in the presence of TCE

F2 *Arabidopsis* and *Sesbania* transgenic plants were cultured in a pot containing 50 gm soil: peat (1:1, V:V) and irrigated in MS liquid medium supplemented with TCE (2%) with three replicates. Wild-type plants of each plant species were similarly treated. All treatments were placed under sterilized greenhouse conditions at 25°C for 16/8 hrs. light/dark photoperiod using day light inflorescents at light a intensity of 3000 lux for 7 days. Plant samples were prepared for analysis by Gas Chromatography (GC), after cleaning and drying with liquid nitrogen to a constant weight and homogenized. The obtained powder (0.3 g for each sample) was pressed into an Eppendorf tube and the samples were transferred to GC section for analysis.

2.2.20.2.3 Extractions of TCE from plant tissues (Doty *et al.*, 2000)

A quantity of 1g tissue was ground with a mortar and pestle and transferred to a chilled glass centrifuge tube. Then 2 ml of 1 M H_2SO_4 and 10% NaCl solution were added to each tube and vigorously shaken for 1 min before 10 ml of *tert*-butyl methyl ether were added. The tubes were then shaken for 1 min, centrifuged at 4°C for 10 min at 8000 xg and 7 ml of each supernatant was transferred to a separate vial containing 2 g of Na_2SO_4. After 1 hr, 1ml samples of the extracts were placed in the GC auto-sampler vials.

2.2.20.2.4 Calibration curve of Trichloroethylene

TCE solutions were prepared at five concentrations (0.000048, 0.00011, 0.0002, 0.004 or 0.01%). The true value was calculated by using rectum line equation (Y= aX + b) which gave sigenificant R^2 (Figure 2.9).

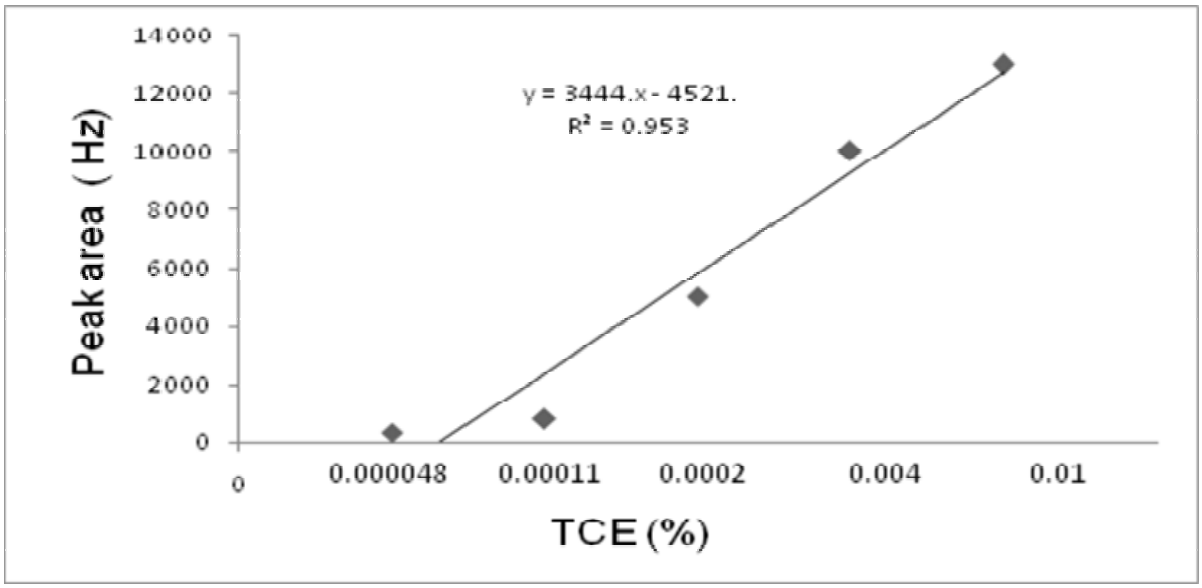

Figure (2.9): Calibration curve of TCE. R^2 value is 0.953.

2.2.21 GC analysis (Doty *et al.*, 2007)

DDT and TCE metabolism was analyzed using GC system which was consisted of a Perkin–Elmer Auto-system gas chromatography equipped with an electron capture detector (ECD) and XTI-5 capillary column (30m x 0.25mm, film thickness 1.0 mm). The oven temperature was ranged from 40°C to 200°C. Helium was the carrier gas, at flow rate of 0.8 ml/minutes Samples of 1 µl were injected automatically in the split mode (split ratio 1:100). Quantitative data were obtained electronically from ECD area, and calibration curves were made for TCE and DDT using commercially available standards (Sigma).

2.2.22 Effect of metals

Stock solutions of metals were prepared by weighing 500 mg of metal salts (KCl, $PbCl_2$, $CuSO_4.5H_2O$, $MnSO_4.\ 5H_2O$) and dissolved in 1L of DDH_2O, then added to the culture medium before autoclaving.

Standard solution (500ppm) with different concentrations of elements {K (200, 250, 300 ppm) Mn (75, 100, 125 ppm) Cu (75, 100, 125 ppm) Pb (30, 40, 50 ppm)} were used in these experiments after mixing with half strength MS medium (without agar). Experiments were performed with the two plant species. Plant samples were prepared for analysis by X-ray fluorescence spectrometer (XRF), by cleaning and drying at 105^{o}C to a constant weight and homogenized. The obtained powder (0.3 g for each sample) was pressed into an Eppendorf tube and the samples transferred to XRF section for analysis.

2.2.22.1 Transgenic experiments in the presence of metals

S. grandiflora and F2 *A. thaliana* transgenic plants were cultured separately in pots containing soil: peat (1:1, V:V) with different heavy metal concentrations as mentioned above with three replicates. Wild type plants of each plant species were treated with the same treatment as the transgenic ones. All treatments were placed under sterilized greenhouse conditions at 25°C for 16/8 hrs. light/dark photoperiod using day light inflorescents at a light intensity of 3000 lux for 12 days.

2.2.22.2 Transgenic experiments in contaminated soil

Samples of contaminated soil were collected from contaminated sites by the AECS qualified staff and analysis for their physicochemical properties (Table 2.5). Samples were put in safety containers and transferred to the

laboratory. Experiments were performed with the two plant species at three periods (5, 10 and 15 days). Plant samples were prepared for metals analysis (Ca, Fe, Br and Zn) by using XRF after cleaning and drying with liquid nitrogen. Then the samples were homogenized, and grounded. The powder (0.3 g for each sample) was pressed into an Eppendorf tubes, samples then taken to XRF examination.

Table (2.5): Some physicochemical properties of mixture (soil:peat) used in these experiments.

Parameter	**Soil texture and values**
Texture	Clay loam
pH	6.3
Total C, g kg^{-1}	59.2
Total organic mater	50%
CEC†, cmolc kg^{-1}	39.1
Total Ca, mg kg^{-1}	5090 ± 388
Total Fe, mg kg^{-1}	2651 ± 120
Total Zn, mg kg^{-1}	42.9 ± 2.6
Total Br, mg kg^{-1}	51.3 ± 2.4

† Cation exchange capacity.

2.2.23 Effect of Uranium

Stock solutions of ^{238}U were prepared by weighing 25 and 50 mM of (UO_2 $(NO_3)_2$) salt and added to the culture medium before autoclaving. Standard solution with different concentrations of uranium 25 and 50 mM was derived and used in these experiments after mixing with half strength of

MS medium (without agar). Experiments were performed with *A. thaliana* plants. Transgenic experiments samples were prepared for analysis by using Gamma analysis, after cleaning and drying with liquid nitrogen then homogenized. The powder (0.3 g for each sample) was pressed into an Eppendorf tubes, samples then taken to Gamma analysis.

2.2.23.1 Germination in different concentrations of Uranium

A. thaliana seeds were germinated separately on MS liquid medium containing different concentrations of uranium (0, 25 and 50) mM with three replicates for each concentration and each replicate included 100 seeds for each plant. Seeds started germinating after 7 days. Germination percentages were recorded after 15 days from sowing. All treatments were placed under sterilized greenhouse conditions 25°C for 16/8 hrs. light/dark photoperiod using day/light inflorescents at a light intensity of 3000 lux.

2.2.23.2 Transgenic plant experiments in the presence of Uranium

F1 and F2 *A. thaliana* transgenic plants were grown separately in pots containing soil: peat (1:1, V: V) supplemented with uranium at 25 mM with three replicates (three pots for each). Each replicate included three pots. Wild type plants of *A. thaliana* were treated as the transgenic. All treatments were placed under sterilized greenhouse conditions at 25°C for 16/8 hrs. light/dark photoperiod using day light inflorescents at a light intensity of 3000 lux for 12 days .

2.2.24 Effect of ^{137}Cesium and ^{90}Strontium

Soils contaminated in ^{137} Cesium 17600 Bq/g and ^{90}Strontium 147 ± 7 mg kg^{-1} were used in the experiments. Experiments were performed with the two plant species for 7days. Plant samples were subjected to analysis as mentioned in 2.2.24.

2.2.24.1 Transgenic experiments in the presence of ^{137}Cesium and ^{90}Strontium

S. grandiflora and F2 *A. thaliana* transgenic plants were grown separately in pots containing ^{90}Strontium 147 ± 7 mg kg^{-1} and ^{137}Cesium 17600 Bq/g with three replicates (three pots for each). Wild type plants of each (*Sesbania* and *Arabidopsis*) were treated in the same manner as the transgenic, and then subjected to environmental condition as mentioned earlier for 7days.

2.2.25 X-ray fluorescence spectrometer (XRF) (Noctor *et al* ., 1996)

Soil samples were ground in tubes (1 ml) with input of silicon carbide for 10 minutes at 30 xg. A sample of 1g was tableted under 15 ton/cm^2 pressure then direct XRF analysis was carried out using calibration curves made with certified reference materials. Correction for mutual multi-element interactions was calculated for calibration curves. The following regression parameters of analytical curves for analysing elements were considered:

Parameters	Analytical curves
K	Y=4.027x +0.041
Ca	Y=2.801x -0.042
Fe	Y=2.055x -0.492
Mn	Y=0.020x +0.021
Cu	Y=0.093x +0.257
Zn	Y=0.093x +0.037
Br	Y=0.191x +0.063
Sr	Y=0.232x +0.093
Pb	Y=0.061x -0.017

2.2.26 Gamma analysis (IAEA, 1999)

A quantity of 10 g of all samples except plants containing Uranium, was bottled in a cylinder (previously-calibrated using mixed gamma solution reference material QCY48), and counted directly for two times. The first time was used to determine ^{238}U, and ^{137}Cs, using HPGe detector P-type R.eff. 80%. The detector is coupled to a MCA and the sample's spectra were analyzed using Interwinner-4 analysis software. Determination of ^{238}U in plant sample was done using a fluorometry technique (technical report series No. 295)

2.2.27 Statistical analysis (SAS/STAT, 2001)

The presented data are an average of three replicates for all experiments except samples used in GC, X- ray and Gamma experiments since they were used as three replicate. A completely randomized design was used. Least significant differences (LSD) were calculated for all data, whereas Qi-square was used only for explants survival rate and significant differences between means were compared. Statistical analysis of data was carried out using SAS package (2001).

Chapter Three
Results
and
Discussion

3 Results and Discussion

3.1 Molecular analysis

3.1.1 *Agrobacterium* regeneration and selection

Results showed that a significant increase in bacterial growth in LB containing Kanamycin and Carbencillin compared to MG/L medium, which was used only for *A. tumefaciens* growth but not for transformation process. This is due to the fact that MG/L contains Mannitol, which cruelly affects transgenic plants and makes them look thin and humid.

A. tumefaciens colonies after 24 hrs of incubation appeared yellow smooth and viscous. Later (after 48 hrs) became somewhat irregular due to viscous fluid secreted by the bacterium.

These results are in agreement with Han *et al.* (2000) who reported that yellow *Agrobacterium* colonies appeared on LB medium containing Kanamycin 50 mg/l and Carbencillin 100 mg/l, but different from that of Doty *et al*. (2007) and Steven *et al*. (2008) who reported a significant increase in *A. tumefaciens* growth on MG/L medium.

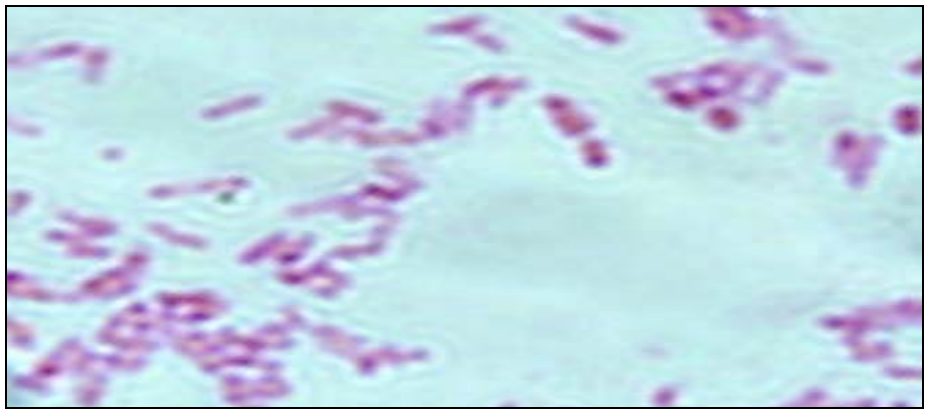

Figure (3.1): *A. tumefaciens* colonies stained with Gram stain 250 X under light microscope. The bacteria were grown on LB medium containing 50 mg/l Kanamycin and 100 mg/l Carbencillin for two days at 25^{o}C.

3.1.2 Effect of Kanamycin resistance on plasmid DNA isolation

pSLD50-6 plasmid containing rabbit CYP2E1 cDNA under the control of the cauliflower mosaic virus 35S promoter was isolated from *A. tumefaciens* grown on LB medium containing two concentrations of Kanamycin (50 and 100 mg/l). Plasmid DNA extraction was carried out using plasmid MiniPrep Kit (QIAgen, Germany) following the manufacturer's instructions. A PCR utilizing two CYP2E1 specific primers was performed to confirm the presence of the gene in the bacterium. DNA electrophoresis (Figure 3.2) confirmed the presence of CYP2E1 (410 bp) in selected *A. tumefaciens* (C58Cl) colonies grown on LB medium containing 50 mg/l Kanamycin.

These results were in harmony with other researchers (Han *et al.*, 2000 and Steven *et al*., 2008) who obtained an equal PCR product

(410bp) when PCRs using identical CYP2E1 primers were performed on *A. tumefaciens* carrying the same plasmid construct.

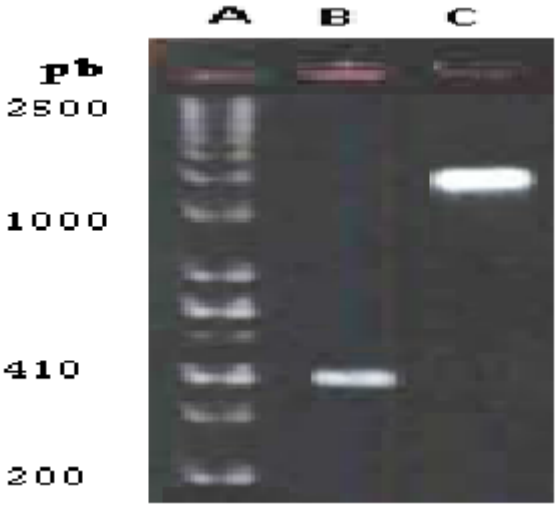

Figure (3.2): Agarose (2%) gel electrophoresis of total DNA extracted from *A. tumefaciens* (70 v, 60 minutes):

A. DNA Leader band of around 2500 – 200 bp
B. PCR fragment of CYP2E1
C. pSLD50-6 plasmid containing rabbit CYP2E1

3.1.3 Primer design and PCR amplification

PCR was successfully amplified *CYP2E1* in all selected *A. tumefaciens* colonies carrying the target construct (positive control) but failed to amplify the same product in both wild types *S. grandiflora* and *A. thaliana* plants (Figure 3.3). Results corresponded with Han *et al*. (2000); Doty *et al.* (2003; 2007) who constructed CYP2E1 plasmid.

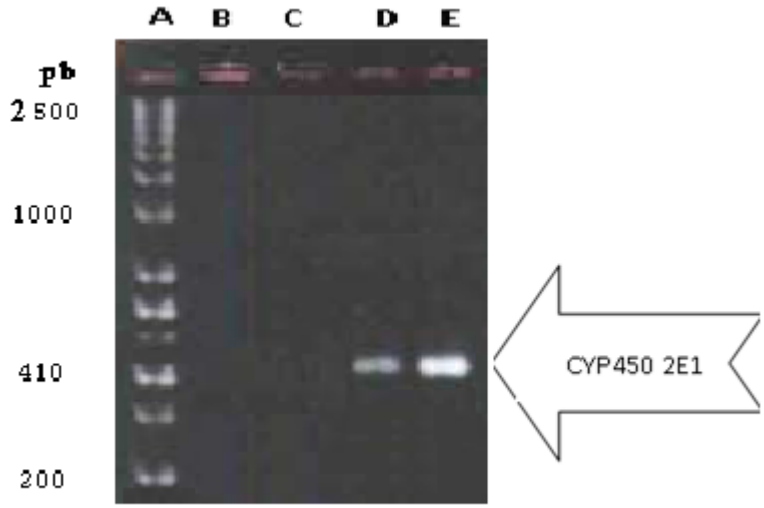

Figure (3.3): Agarose (2%) gel electrophoresis of PCR amplicons using CYP2E1 specific primers (70 v, 60 minutes):

A. DNA Leader band of around 2500 – 200 bp
B. Wild type *S. grandiflora*
C. Wild type *A. thaliana*
D and E. *A. tumefaciens* DNA containing CYP2E1.

3.1.4 Germination of seeds

Results showed that the percentage of germination on MS medium after 10 days was 50%. However, when seeds were inoculated on sterilized moistened filter paper, the germination percentage reached 100% after 5 days of sowing (Figure 3.4).

In this experiment, the percentage of seed germination on the solidified medium was low compared with moistened sterilized filter. This might be due to impurities in the agar and/or to the osmotic potential (Bewley and Black, 1994 and Agarwal *et al.,* 1997). A high percentage of seed germination has been reported on sterilized moistened filter paper in various crop species, including *A. thaliana* seeds (Polisetty *et al.,* 1997). Thus, in all

experiments, seeds were germinated on sterile moistened filter paper.

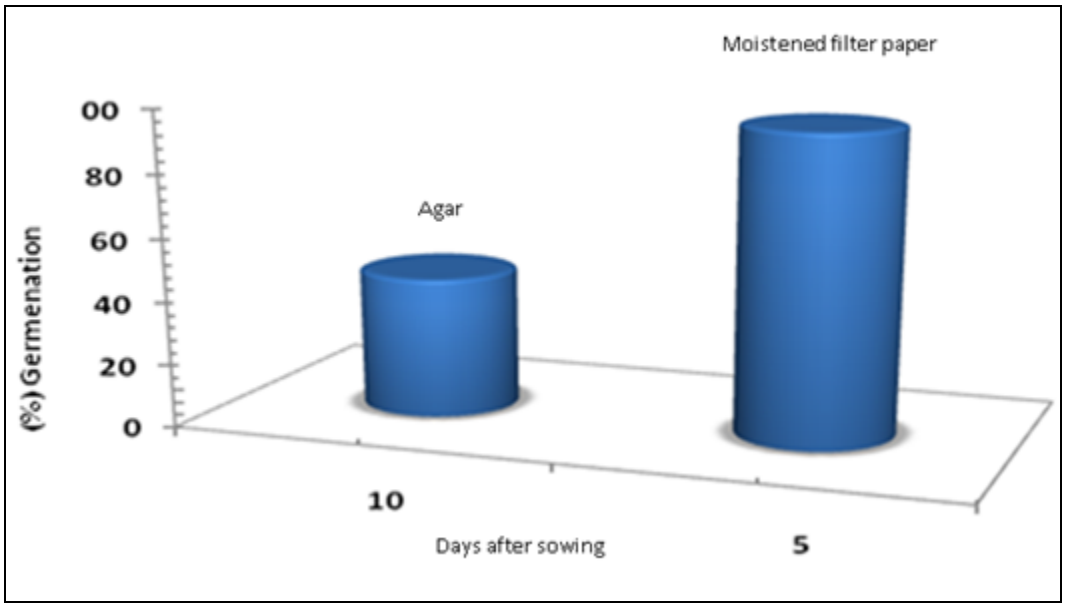

Figure (3.4): *S. grandiflora* and *A. thaliana* seeds germinated on half strength MS medium solidified with 7 g/l agar or on moistened sterile filter paper. Both plants are identical in their % germination.

3.1.5 Determination of Kanamycin resistance

S. grandiflora were germinated on half strength MS medium containing different concentrations of Kanamycin (0, 50, 100 or 150 mg/l) (Figure 3.5). After 10 days, all seeds germinated and cotyledons looked healthy. The frequency of germination was found to be 94% at 50 mg/l Kanamycin, 88% at 100 mg/l Kanamycin and 80% at 150 mg/l Kanamycin. Siliques were bleached and then died after three weeks of inoculation regardless of the Kanamycin concentration except that inoculated on half strength MS medium supplemented with 50 mg/l Kanamycin. This clearly indicates that 50 mg/l Kanamycin is the convenient concentration at which selection of

transgenic plants was performed. Other Kanamycin concentrations, 100 and 150 mg/l were not useful for transformation process.

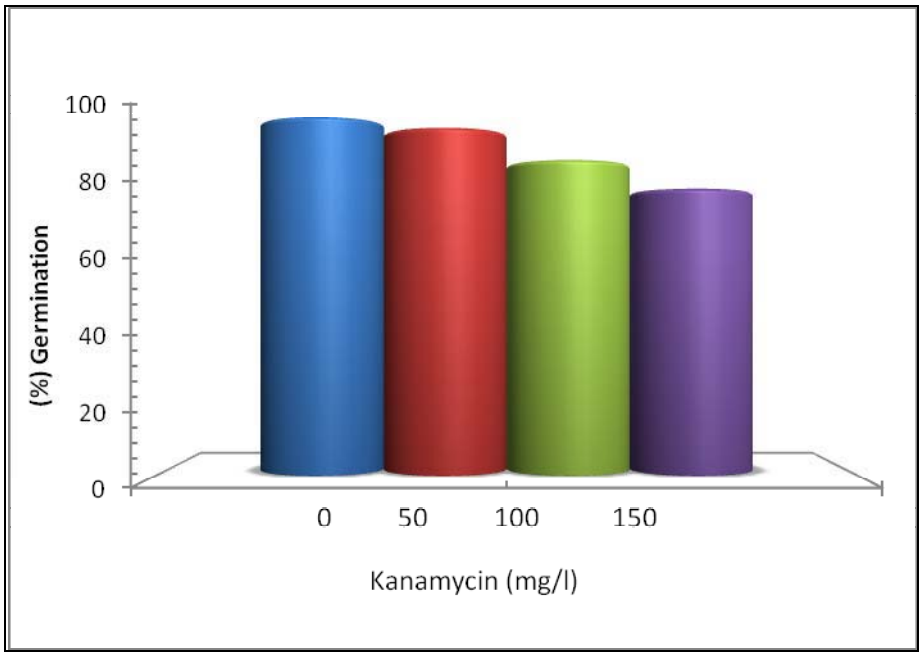

Figure (3.5): *S. grandiflora* and *A. thaliana* seeds germinated on half strength MS medium containing different concentrations of Kanamycin for 10 days. Both plants are identical in their % germination.

These results were in agreement with Doty *et al.* (2000; 2007) who reported that 50 mg/l Kanamycin was suitable for transformants with *A. tumefaciens.*

3.1.6 Transformation

The introduction of rabbit *CYP 2E1* into a plant genomes and the regeneration process are influenced by various factors that were studied.

3.1.6.1 Inoculation and selective media

Prior to transformation, an effective concentration of antibiotic for selection and regeneration of transformed cell was determined by culturing

cotyledons on MS medium containing 50 mg/l Kanamycin, while mixtures of liquid MS and LB medium; MS and MG/L medium were used for explants infection.

Results showed that the combination between MS and LB medium (15: 5) was useful for transformation in comparison with MS and MG/L medium (15: 5). MG/L medium containing mannitol causes explant tissues to become water- soaked then show severe leaf necrosis ended with explant death within 2-4 days after inoculation (Figure 3.6).

Steven and Andrew (1998) reported that explant tissues become necrotic and viscous when a combination of MS and MG/L medium was used as an infection medium with *A. tumefaciens*.

On the other hand, Doty *et al.* (1996; 2007) reported that MS and MG/L medium was suitable for *A. tumefaciens* growth and infection of Poplar explants.

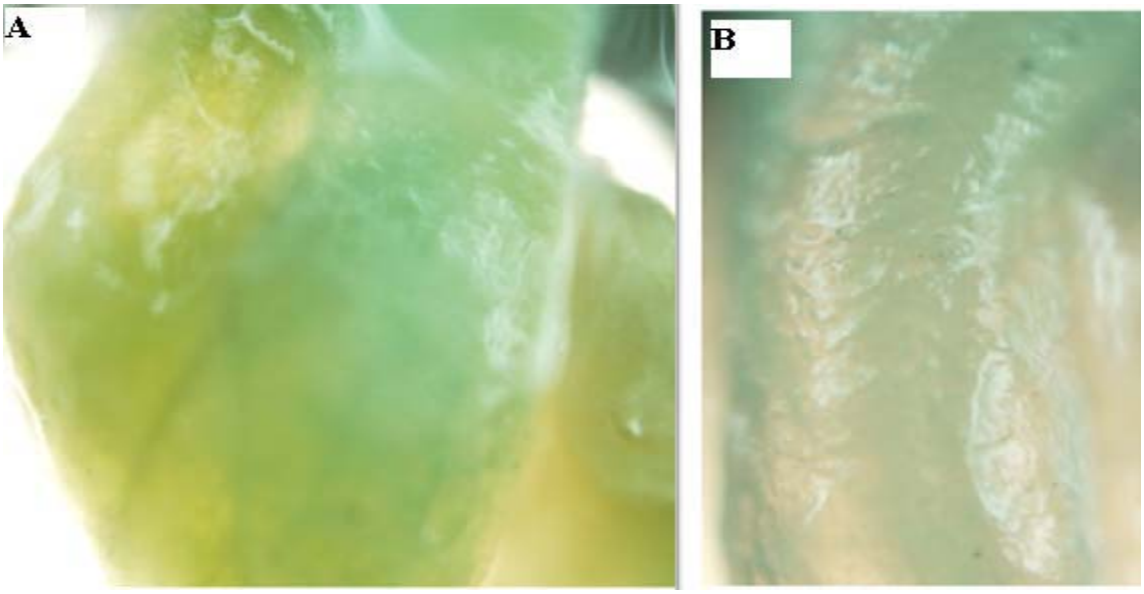

Figure (3.6): *S. grandiflora* explants appeared watery (A) and viscous (B) after 2-4 days of inoculation with *A. tumefaciens* on MG/L medium.

3.1.6.2 Wounding of explants

Acetosyringone is a phenolic compound, which is secreted at wounded site of *S. grandiflora* cotyledon explants. This compound enhances *Agrobacterium*–mediated gene transformation in dicots through the activation of certain virulence genes (*Vir*). This consequently leads to the synthesis of a single-stranded T-DNA carrying the gene of interest. Other *Vir* genes will initiate the transfer and the integration of this T-DNA into the host nuclear genome. Veluthamble *et al.* (1989) reported that the addition of acetosyringone increases transformation efficiency.

In this study wounding played a critical role in transformation efficiency. Additionally, Hiei *et al.* (2004) reported that acetosyringone induces *Vir* genes, extending host range of some *Agrobacterium* strains such as C58Cl.

3.1.6.3 Exposure time for infection

Exposure time required for infection is one of the main factors among others affecting *A. tumefaciens* mediated gene transformation.

Results showed that an increased time of infection led to a significant decrease in explant regeneration. An infection period of 30 minutes was critical for the regeneration process. While, 40 and 45 minutes infection periods decreased regeneration efficiency and resulted in solid and viscous explants unable to grow and regenerate (Figure 3.7). This might be due to the fact that the infection medium is acidic (pH 4.7-4.8) which may increase osmotic pressure.

These results are confirmatory of Rashied *et al.* (1996) and Cho *et al.* (2004).

Figure (3.7): *S. grandiflora* explants appeared viscous and contracted after 35 (A) and 40 (B) minutes of infection with *A. tumefaciens.*

3.1.6.4 Callus initiation

Cotyledonary explants were used for the regeneration of *S. grandiflora*. After one week of inoculation, the size of the cotyledons increased and callus started initiation at the cut ends of the explant.

Wild type tissues appeared brown, while transformed tissues displayed healthy growth on MS medium containing 400 mg/l Carbencillin and 50 mg/l Kanamycin (Figure 3.8). This is due to the presence of two antibiotics, which killed wild type tissues (non-transformed) that could not survive on the selective medium.

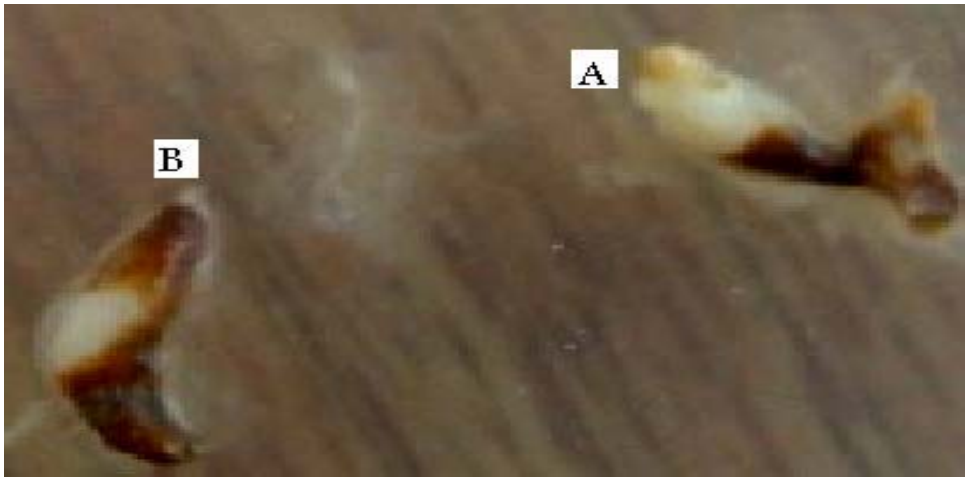

Figure (3.8): Effect of Carbencillin (400 mg/l) and Kanamycin (50 mg/l) on transformed (A) and non transformed (B) explants cultured on MS medium.

Explants were recultured on MS medium containing 400 mg/l Carbencillin and 50 mg/l Kanamycin, at 10 day intervals to prevent growth of wild type cells. Figure 3.9 shows the induction of callogenesis (shoot formation). Cells started to elongate. Shoot buds and shoots started

originating via indirect organogenesis from the tissue around the cut edges after 14–15 days and callus had the ability to either proliferate or differentiate.

Figure (3.9): Growth and elongation of embryogenic calli on MS medium containing 400 mg/l Carbencillin and 50 mg/l Kanamycin.

3.1.6.5 Plant regeneration from callogenisis transgenic cell

Results showed in tables 3.1; 3.2 indicat that MS medium supplemented with 0.5 mg/l BAP plus 0.2 mg/l IAA; 1.5 mg/l BAP plus 0.5 mg/l NAA gave the highest rate of shoot formation percentage reaching 76 and 60% respectively compared with 33% and 23% when the same medium was supplemented with 1.5 mg/l BAP plus 0.5 mg/l IAA; 0.5 mg/l BAP; 0.1 mg/l NAA respectively.

At these concentrations, there was no response at all in the control treatment. Reducing combinations of plant growth regulators produced green healthy long shoots whereas the higher concentration of BAP and IAA produced glossy shoots.

High concentrations of BAP resulted in increasing the frequency of vitrification (glassiness) is usually unsuitable for further culture.

Table (3.1): Effect of BAP and IAA on shoot regeneration from cotyledonary explants of *S. grandiflora* after 4 weeks in culture.

BAP (mg/l)	**IAA (mg/l)**	**Shoot formation (%)**
0.0	0.0	*
0.5	0.2	76
1.5	0.5	33
Mean		54.5
$LSD_{0.05}$		8.60

*No response for shoot formation

Table (3.2): Effect of BAP and NAA on shoot regeneration from cotyledonary explants of *S. grandiflora* after 4 weeks in culture.

BAP (mg/l)	**NAA (mg/l)**	**Shoot formation (%)**
0.0	0.0	*
0.5	0.1	23
1.5	0.5	60
Mean		41.5
$LSD_{0.05}$		5.4

*No response for shoot formation

These results are similar to that of Allavena and Sarp (1981) who induced multiple and adventitious shoots on beans in a basal MS medium supplemented with 1.5 mg/l BAP in combination with 0.5 mg/l IAA or NAA.

Moreover, Nandwani and Ramawal (1992) reported that high frequency of plantlet regeneration from cotyledons of Soybean was obtained on MS medium supplemented with IAA and BAP. In the same context, Ashis *et al.* (2007) reported that tobacco plants could be easily regenerated from leaf pieces on MS medium supplemented with 1 mg/l BAP and 0.1mg/l NAA.

Table 3.3 showed that the effect of another cytokinin, 0.5 mg/l KIN coupled with 0.1 mg/l IAA achieved 80% response. However, when concentrations increased to 1.5 mg/l KIN and 0.2 mg/l of IAA, a 32% response was obtained. While in the control treatment no shoot formation response was seen. It was found that the increasing the combination concentrations, the lower percentage of shoot regeneration and vice versa.

KIN and NAA (Table 3.4) achieved 68% response in the combination of 1.5 mg/l KIN and 0.2 mg/l of NAA, and 18% in the combination of 0.5mg/l KIN and 0.1 mg/l of NAA, while the control treatment showed no shoot response

In this study, the effects of BAP and IAA combinations on shoot organogenesis in *S. grandiflora* were analyzed after *A. tumefaciens* transformation. A combination of BAP and IAA has been most often used for shoot organogenesis in legume and was also reported to be effective for shoot organogenesis in other plants.

Table (3.3): Effect of KIN and IAA on shoot regeneration from cotyledonary explants of *S. grandiflora* after 4 weeks in culture.

KIN (mg/l)	**IAA (mg/l)**	**Shoot formation (%)**
0.0	0.0	*
0.5	0.1	80
1.5	0.2	32
Mean		56
$LSD_{0.05}$		5.40

*No response for shoot formation

Table (3.4): Effect of KIN and NAA on shoot regeneration from cotyledonary explants of *S. grandiflora* after 4 weeks in culture.

KIN(mg/l)	**NAA (mg/l)**	**Shoot formation (%)**
0.0	0.0	*
0.5	0.1	18
1.5	0.2	68
Mean		43
$LSD_{0.05}$		6.30

*No response for shoot formation

3.1.6.6 Rooting induction

Table 3.5 shows that IBA addition at three concentrations (0.05, 0.1 or 0.2 mg/l) led to 62, 70 and 59% increases in the percentage of root regeneration respectively. In contrast, the control treatment showed no root regeneration response.

Similarly, IAA (table 3.6) added at 0.05, 0.1 or 0.2 mg/l led to significant increases 75, 100 and 84 in the percentage of root regeneration respectively. Control treatment showed no root regeneration. In comparison, 0.1 mg/l of IAA gave 100% root regeneration, while the same concentration of IBA gave only 70% root regeneration. Over time, roots elongated and put out numerous thin laterals. Plantlets were then hardened and fully expanded leaves and well-developed roots were achieved.

Similar results were observed by Desal and Halepyati (2007) who reported that IAA is an efficient growth regulator for rooting. In this work, IAA was found to be the best auxin for rooting since 100% of shoots were rooted with 0.1 mg/l IAA.

Figure (3.5): Effect of IBA on rooting of *S. grandiflora* shoots after 6 weeks in culture.

IBA (mg/l)	**Rooting (%)**
0.0	*
0.05	62
0.1	70
0.2	59
Mean	62.66
$LSD_{0.05}$	4.631

*No rooting response

Figure (3.6): Effect of IAA on rooting of *S. grandiflora* shoots after 6 weeks in culture.

IAA (mg/l)	**Rooting (%)**
0.0	*
0.05	75
0.1	100
0.2	84
Mean	86.33
LSD $_{0.05}$	2.681

*No rooting response

3.1.7 Transformation of *A. thaliana* floral by dipping

Floral dipping methods have made it possible to transform *A. thaliana* with no need for plant tissue culture or regeneration techniques (Figure 3.10). Culturing media, hormones and pH adjustment were unnecessary and *Agrobacterium* mediated transformation is applicable at a range of cell densities. *A. thaliana* plants were infected with the same *A. tumefaciens* strain using the floral dip method. Plants were dipped in a solution of *A. tumefaciens*, 5% sucrose and 0.02% of the surfactant, Silwet L-77. Plants were inoculated when numerous immature floral buds and few siliques were present. Plants were left to set up seeds and transgenic seeds were selected on Kanamycin containing medium.

Floral dip method is simple can be conducted by submerging the above-ground parts of the plant into an *Agrobacterium* solution for a few seconds. In nature, wounded sites secrete compounds (hexoses at low pH) that induce *vir* genes. In tissue culture transformation methods, the phenolic compound

acetosyringone is sometimes added to induce expression of relevant *vir* genes. Bechtold *et al*. (1993) and Al-Daoude (2003) reported that *A. tumefaciens* C58Cl and pTiC56 genotypes are effective in *A. thaliana* transformation.

Steven *et al*. (2008) reported that an increase in Silwet L-77 from 0.005% to 0.1% gave higher transformation rate, but high level surfactant (Silwet L-77) can cause plant tissue necrosis under various conditions.

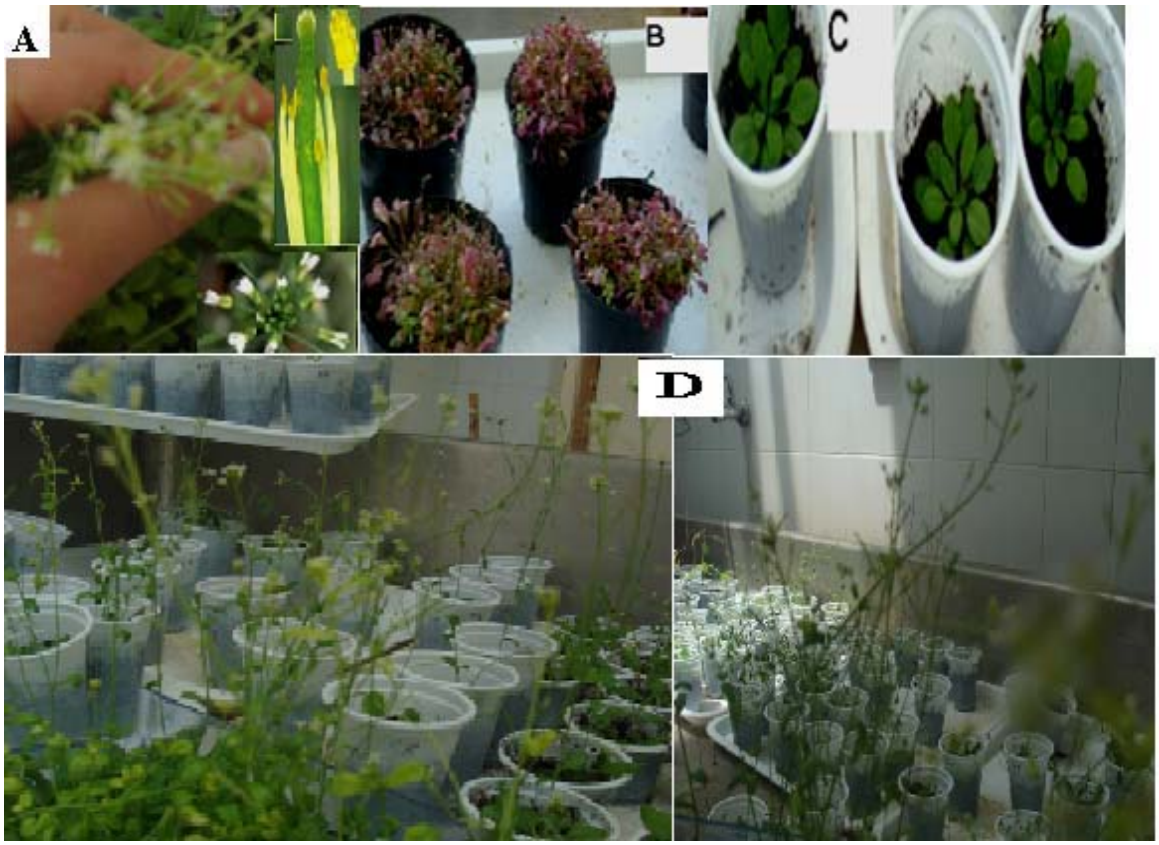

Figure (3.10): *A. thaliana* growth stages with floral dipping method:

A. Primary bolts clipped, secondary bolts about 2-10 cm (few open flowers)
B. *A. thaliana* clusters were harvested after floral dipping with *A. tumefaciens*.
C. *A. thaliana* germinated on soil containing 50 mg/l Kanamycin.
D. *A. thaliana* F1 progeny was harvested and seeds were germinated under Kanamycin selection.

3.1.7.1 Plant growth stage

One of the most important variables present in the transformation protocol is the developmental stage of the plant at the time of inoculation with *Agrobacterium*. For identify the inflorescence developmental stage that is most suitable to transformation, primary bolts were clipped allowing the rise of many secondary bolts.

This result is supported by Steven and Andrew (1998) who tested the suitability of different growth stages for floral dipping. They examined first, plants with primary bolts clipped and secondary bolts about 1-5 cm, second plants with secondary bolts about 2-10 cm (few open flowers) and third plants with non clipped primary bolts and many open flowers starting to produce siliques and finally plants with mature bolts and many developed siliques. They confirmed that the highest transformation rate was obtained when plants from the second group were used and plants in the last group gave the least transformation rate (Al-Daoude, 2003; Al-Daoude *et al.* 2005).

3.1.7.2 Simplification of the inoculation medium

A. tumefaciens cells were grown in a medium with pH 5.7, harvested by centrifugation and then suspended in an inoculation solution consisting of MS medium containing 5% sucrose and 0.02% Silwet L-77 that was used to reduce surface tension, which greatly enhances bacteria entry into relatively inaccessible plant tissues. Similar observations were reported by Whalen *et al.* (1991), when they stated that there are three main requirements for a successful transformation procedure starting with the correct plant developmental stage, sugar type and type of surfactants used.

3.1.8 Detection of the transgenic using PCR

Polymerase Chain Reaction (PCR) was carried out in order to confirm the presence of CYP2E1 gene in transgenic plants.

Genomic DNA was extracted from plantlet leaves and was used to perform a PCR utilizing two CYP2E1 specific primers. PCR amplicons were separated on 2% agraose gels and photographed under UV light (Figure 3.11 and Figure 3.12). The process was repeated several times until enough numbers of plantlets needed for chemical experimentations was obtained. Selected transgenic plantlets were then acclimatized.

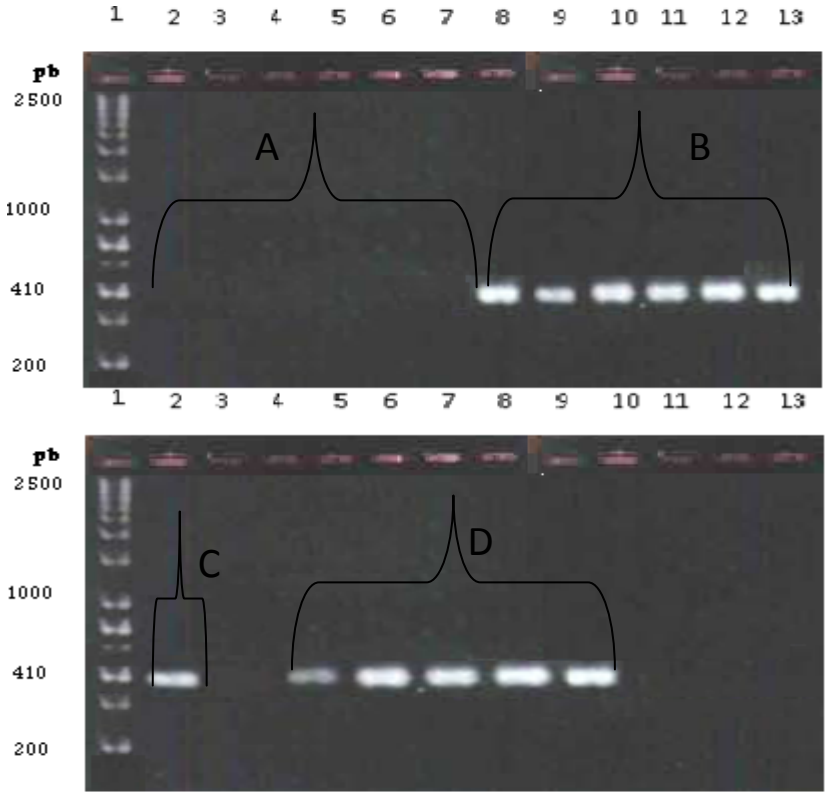

Figure (3.11): Agarose (2%) gel electrophoresis of PCR amplicons using CYP2E1 specific primer (70 v, 60 minutes). Lane 1: DNA Ladder (Band of around 2500 – 200 bp). Ninety six DNA samples were extracted and tested for the presence of CYP2E1 gene (a band of ~410 bp was seen).

A. Control plants; B, C and D are transgenic plantlets.

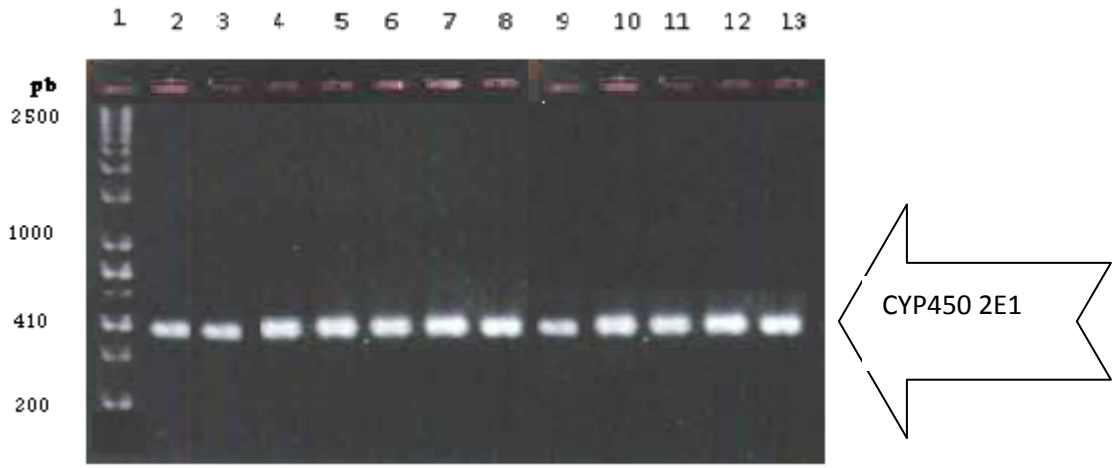

Figure (3.12): Agarose (2%) gel electrophoresis of PCR amplicons using CYP2E1 specific primer (70 v, 60 minutes), DNA Ladder band of around 2500 – 200 bp. Twenty four DNA samples were seen with bands around 410 bp (CYP2E1 gene).

3.1.9 Western blotting

S. grandiflora and *A. thaliana* total protein extracts were obtained using standard methods (Natalia *et al.,* 2002). The ECL method was used for visualization of protein following the manufacturer's recommendations (Amersham, USA) (Figure 3.13). gene expression in *S. grandiflora* transgenic plants was much higher than that detected in *A. thaliana* transgenic plants, which was reversed on western blotting (Figure 3.13).

Natalia *et al.* (2002) reported similar observation with mice. These results were in the same vein as in Doty *et al.* (2000; 2007) and Doty (2008) who reported similar observations with the *Poplus spp*.

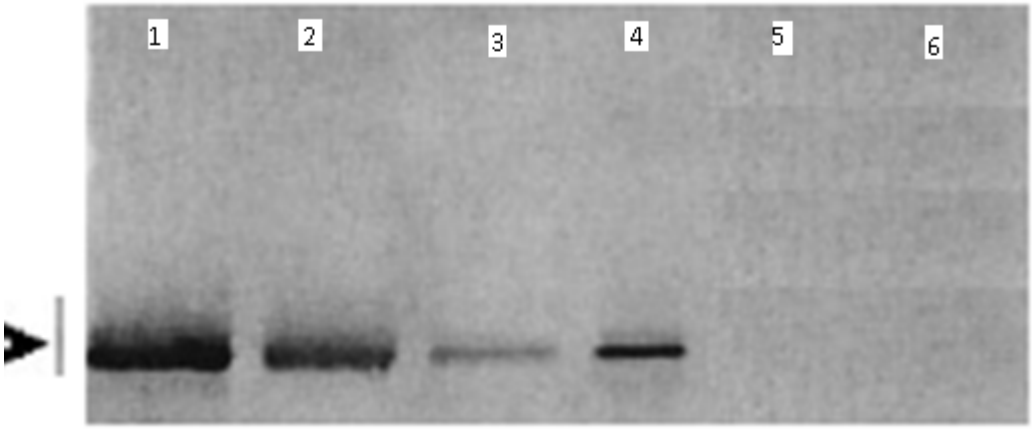

Figure (3.13): Detection of CYP2E1 in transgenic *A. thaliana* and *S. grandiflora* plants using western blotting technique.

Lanes 1 and 2 are two independent *S. grandiflora* transformants.

Lanes 3 and 4 are two independent *A. thaliana* transgenic lines.

Line 5 is the wild type of *A. thaliana* plants.

Line 6 is the wild type of *S. grandiflora* plants.

3.2 Effect of pollutants

3.2.1 Dichlorodiphenyltrichloroethane (DDT)

Analysis of DDT, DDE and DDD extracted from *A. thaliana* and *S. grandiflora* plants were conducted (Table 3.7). The identification was confirmed by using standards and comparison with data published in the literature (Rsoc, 1999). Retention times were 23.351, 22.876 and 20.489 for DDT, DDD and DDE respectively (Table 3.7).

Table (3.7): Retention time of DDT, DDD and DDE analyzed by GC-ECD.

Compound	**Retention time**
DDT	23.351
DDD	22.876
DDE	20.489

3.2.1.1 Germination of *S. grandiflora* and *A. thaliana* seeds in the presence of DDT

At the end of day 15, germination percentage reached 99.3, 60.6, and 20.6% for the DDT concentrations 0, 10, and 20 μg/ml respectively (Figure 3.14).

There was no direct relationship between DDT uptake by the seeds and its subsequent translocation to the growing regions or the degree of growth inhibition. Data suggest that oil content of the seeds affect the susceptibility or tolerance of a plant to DDT. It is suggested that lipids of the

plant cell solubilize and disperse DDT in the cytoplasm, which, in turn, affects normal metabolism within the cell (Bumpus and Aust, 1987).

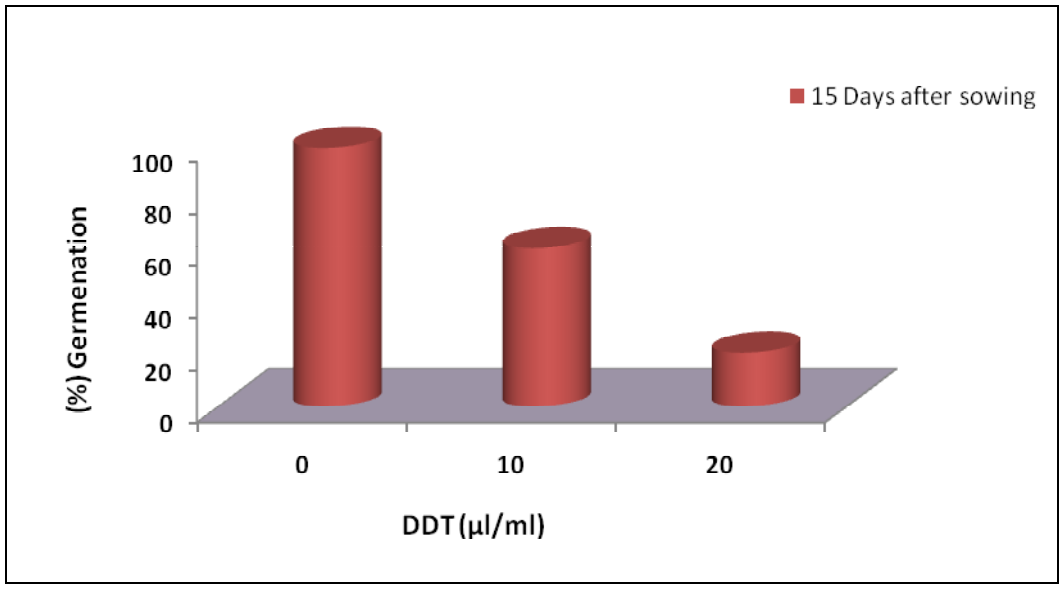

Figure (3.14): Germination percentages of *S. grandiflora* and *A. thaliana* seeds after 15 days of sowing on different DDT concentrations. Both plants behaved similarly.

Rice (*Oryza sativa*) plants expressing enzymes that degrade herbicides may be helpful in reducing the load of herbicides in paddy fields and streams. Transgenic rice with *CYP2B6* germinated well on a medium containing 2.5 µ l/ml alachlor or 5 µl/ml metolachlor, while non-transgenic rice could grow at all. The *CYP2B6* rice plants also grow on a medium with herbicides as on herbicide free medium (Hirose *et al*., 2005).

3.2.1.2 Metabolism of DDT

CYP2E1-containing transgenic plants increased the average of DDT metabolism that was nearly 85-fold greater than that expressed in the wild type (Figure 3.15 and 3.16). Transgenic plants grew normally and displayed no adverse reaction to added DDT.

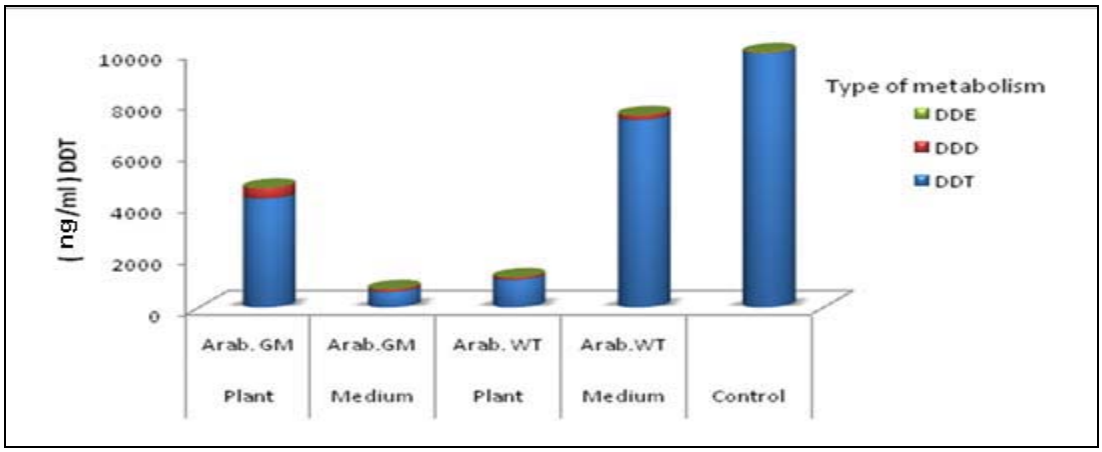

Figure (3.15): Concentration of DDT and its metabolism to DDD, DDE in *A. thaliana* plants and culturing medium after 12 days.

Arab. WT = *A. thaliana* wild type plants

Arab. GM = *A. thaliana* transgenic lines

Control = Untreated medium.

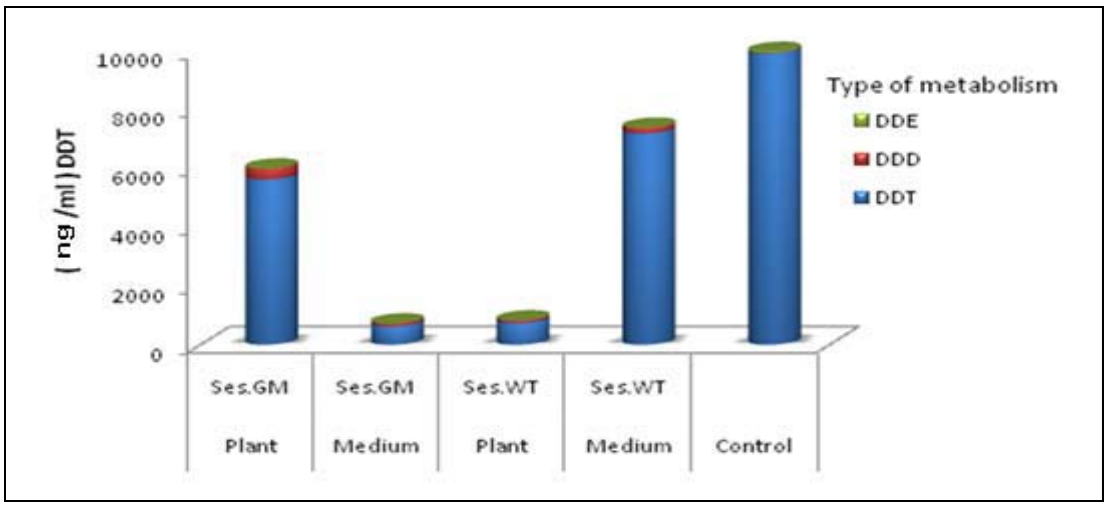

Figure (3.16): Concentration of DDT and its metabolism to DDD, DDE in *S. grandiflora* plants and culturing medium after 12 days.

Ses. WT = *S. grandiflora* Wild type plants

Ses. GM = *S. grandiflora* transgenic lines

Control = Untreated medium

Similar trend was also exhibited for DDD and DDE. This elucidates the grate ability of CYP2E1 to express itself in the new host plants increasing their capability in removing contaminants.

To study whether the increased metabolism of DDT by transgenic *S. grandiflora* and *A. thaliana* plants is due to the transgene, the concentration of DDT in the solution was monitored. CYP2E1 transgenic plants removed DDT from solution at much greater rates than control once. After 12 days, wild type grown on untreated medium (control) removed 3% of the added DDT from solution.

Transgenic plants removed about 91% of the DDT present in the culture medium. Calculation the percent removal of DDT from solution showed that *S. grandiflora* and *A. thaliana* lines are significantly different from their controls. The best performing transgenic *A. thaliana* removed DDT 53 times faster more wild type plants.

Other workers showed that wild type plants and microorganisms, such as *Pleurotus luteoablus* degrade 69% of DDT, and 3% of which was mineralized during 30 days (Pflugmacher and Sandermann, 1992; Sandermann, 1992). In another study, (Sandermann, 1994) the first intermediate compound of the insecticide DDT metabolism in soybean was shown to be conjugated by the formation of O-glucoside. Conjugation capacity of soybean Oglucosyltransferase is 855µg DDT per hr per g fresh weight (Sandermann, 1994). Other species like *Phanerochaete chrisosporium, Phanerochaete sordid, Phellinus werii, Polyporus versicolo, Pleurotus ostreatus* were reported to degrade ~50% DDT in 30 days, with 5–14% of DDT mineralized to CO_2 (Bumpus and Aust, 1987; Safferman *et al*., 1995).

S. grandiflora and *A. thaliana* transgenic plants grown on DDT containing soil were healthy 12 days after the start of experiment of work and have higher fresh and dry weight than wild type plants (Table 3.8).

Table (3.8): DDT uptake, fresh weight and dry weight in transgenic and wild type plants.

Type of plant	DDT uptake (ng/ml)	Fresh weight (g)	Dry weight (g)
A. thaliana WT	1081	0.333	0.017
A. thaliana GM	4255	0.923	0.07
S. grandiflora WT	744.49	1.015	0.012
S. grandiflora GM	5643.51	3.112	0.024

DDT uptake was 1081ng/ml and 744.49ng/ml in wild type *A. thaliana* and *S. grandiflora* plants respectively. In contrast, the highest DDT uptake (5643.51ng/ml) was recorded in transgenic *S. grandiflora* plants compared with 4255ng/ml in transgenic *A. thaliana* plants. The highest fresh weight (3.112 g) was recorded in transgenic *S. grandiflora* plants while wild type plants recorded 1.015g. The lowest fresh weight (0.333g) was recorded in wild type *A. thaliana* while transgenic *A. thaliana* gave 0.923g. The lowest dry weight (0.017 g) was recorded in wild type *A. thaliana* while transgenic *A. thaliana* gave 0.07g. The highest dry weight (0.024g) was observed in *S. grandiflora* transgenic plants compared with wild type plants (0.012g).

A. thaliana plants achieved higher uptake and lower fresh weight compared with the *S. grandiflora* plants. This could be due to that *A. thaliana* plants have shorter life cycle and a large rooting system.

Phytoremediation of organic pollutants involves the biochemical decomposition of foreign chemicals (xenobiotics) in plant tissues. Metabolism of xenobiotics in plants usually takes place in three phases. In Phase I, xenobiotics may undergo hydrolysis, reduction, or oxidation transformations (Doty *et al.,* 2005). Phase I enzymes are also involved in a number of reductive reactions under oxygen-deficiency conditions (Doty, 2008).

Typically, these reactions result in the introduction of functional groups in the molecule or the exposure of preexisting functional groups and leads to the formation of more polar, more water-soluble, and chemically more reactive and sometimes biologically more active derivatives. Therefore, Phase I is often called the bioactivation phase of metabolism. In Phase II, the actual detoxification step happens where the product from Phase I is bound to an endogenous sugar of peptide molecule. Phase III represents the final removal and decomposition of these conjugates *via* exporting them into the cell vacuole, and/or incorporating them into biopolymers, such as lignin. Compartmentalization is a potential final step in the non-oxidative utilization of xenobiotics, by storage in certain compartments of the plant cell. Soluble conjugates (peptides, sugars, amino acids etc.) are accumulated in vacuoles, insoluble conjugates (coupled with pectin, lignin, xylem and other polysaccarides) are being out of the cell and accumulated in plant cell wall (Sandermann, 1994). The insecticide DDT acquires a carboxyl group by primary oxidation, then turns into ether with glucose using this carboxyl group and the formed conjugate is stored in the vacuole (Pierrel *et al*., 1994; Robineau *et al*., 1998).

3.2.2 Trichloroethylene

Chemical analysis of TCE and metabolism to Chloral and Trichloroethanol extracted from *A. thaliana* and *S. grandiflora* plants were conducted (Table 3.9). The identification was confirmed by standards then compared with data published in the literature (Doty *et al*., 2007).

Table (3.9): Retention time of Trichloroethane, Chloral and Trichloroethanol as determined by GC-ECD.

Compound	**Retention time**
TCE	2.92
Chloral	3.301
Trichloroethanol	4.942

Table 3.9 shows that the retention times were 2.92, 3.301 and 4.942 for Trichloroethane, Chloral and Trichloroethanol respectively. TCE oxidation by P450 2E1 is chloral (2,2,2-trichloroacetaldehyde), which is further metabolized to Trichloroethanol or Trichloroacetic acid .

This result is in agreement with Doty *et al*. (2007) who suggested that *P450 2E1* gene in tobacco cell roots has metabolized TCE to Chloral and Trichloroethanol.

3.2.2.1 Germination of *S. grandiflora* and *A. thaliana* seeds in the presence of TCE

At the end of day 15, germination percentages reached 99.3, 60.7, and 20.9% at TCE concentrations 0, 2, and 4% respectively (Figure 3.17).

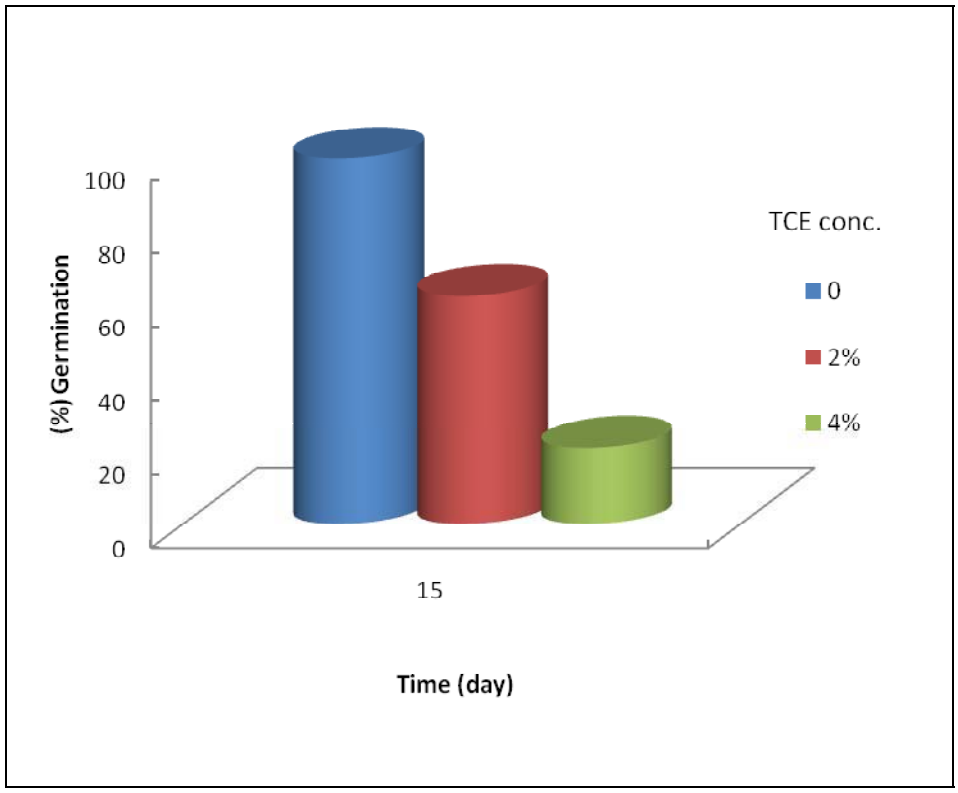

Figure (3.17): Germination percentages of *S. grandiflora* and *A. thaliana* seeds after 15 days of sowing on different TCE concentrations. Both plants behaved similarly.

While it is important to understand the concentrations of TCE that are toxic to plants, few studies report the phytotoxic effects of TCE. Gordon *et al*. (1998) reported that poplars are able to survive in water containing 0.5% of TCE. Another experiment found that TCE is toxic to a variety of crop plants at a concentration of 0.2% in the gas phase. The later study hypothesized that an increase in electrolyte leakage or interference with the photosynthetic system was the mechanism of acute toxicity in the plants, but the exact mechanism is not known.

3.2.2.2 Metabolism of TCE

Transgenic *A. thaliana* and *S. grandiflora* plants showed an average increase in TCE metabolism that was nearly 75 fold greater than that detected in respective wild types. Moreover, transgenic plants grew normally and did not display any adverse reaction to TCE presence or its metabolites.

It is clear from the results shown in figure 3.18 that transgenic *A. thaliana* reduced TCE to 4.5g/l compared with wild type plants which accumulated 14.0g/l. Trangenic *A. thaliana* plants accumulated TCE 3.11 times higher than that deteced in wild type. TCE metabolism to Chloral was 6.7g/l and 5.96g/l in transgenic and wild type plants respectively. Similarty TCE metabolism to TCEOH was 1.81g/l comapred with 0.91g/l in transgenic and wild type plants respectively.

TCE uptake in transgenic *S. grandiflora* increased to 13.92g/l compared with 2.17g/l in wild types. TCE metalbolism to chloral was 0.71g/l and 1.28g/l in transgenic and wild type *S. grandiflora* plants resepectively. However, higher conversion of TCE to TCEOH was recoreded in transgenic *S. grandiflora* (5.05g/l) compared with the wild type (2.89g/l).

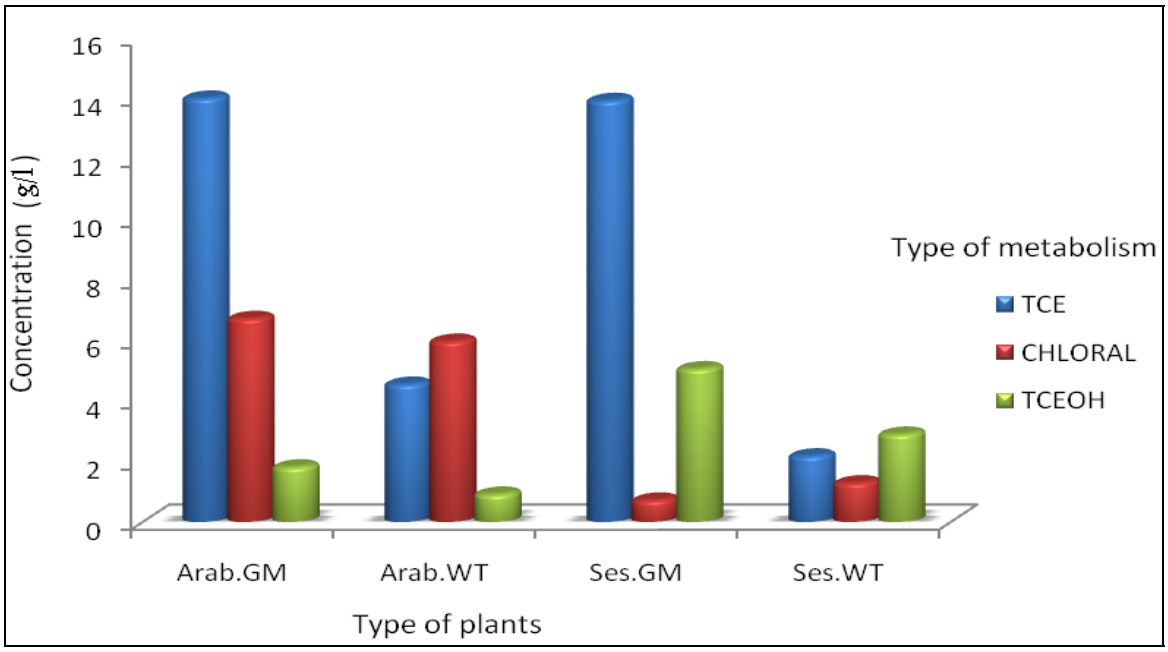

Figure (3.18): TCE Concentration (g/l) and its metabolism to Chloral, and Trichloroethanol in transgenic (GM) and wild type (WT) *A. thaliana* and *S. grandiflora* plants after 7days.

Table 3.10 shows that the highest TCE uptake (13.9g/l) was recorded in transgenic *S. grandiflora* plant while *S. grandiflora* wild plants uptake 2.17g/l only. Transgenic *A. thaliana* plants absorbed 14g/l TCE compared with 4.54g/l in wild type. The highest fresh weight (3.415g) was reached in transgenic *S. grandiflora* compared with 2.012g in the control plants. The lowest fresh weight (0.343g) was recorded in *A. thaliana* wild type compared with the transgenic one (0.523g).

A. thaliana plants gained the highest uptake and the lowest fresh weight than *S. grandiflora* plants. This could be due to the fact that *A. thaliana* plants have shorter life cycle and larger rooting systems.

Additionally, the TCE toxic effects (low level of photosynthesis and leaves turning yellow) were observed only on both wild-type plants but not on transgenics.

Table (3.10): TCE uptake and fresh weight in transgenic and wild type plants.

Type of plant	TCE uptake (g/l)	Fresh weight (g)
A. thaliana WT	4.54	0.343
A. thaliana GM	14.0	0.523
S. grandiflora WT	2.17	2.012
S. grandiflora GM	13.9	3.415

It is important to understand the upmost concentrations of TCE that are toxic to plants; few studies reported the phytotoxic effects of TCE. For a plant to directly degrade, mineralize, or volatilize a compound, it must be able to absorb that compound up through its roots. Phytoremediation appears to be a valuable option for treating dissolved TCE in ground water and soils, on the other hand, pose a potential problem for plants since they may contain high levels of organic matter favor more polar molecules because of the competing process of absorption to soil organic matter. Plant roots may have a difficulty in extracting TCE from soils containing significant amounts of organic matter.

In this study the importance of Phase I type biotransformation are reviewed. Phase I reactions are most important in the phytoremediation of hydrophobic, chemically stable pollutants, such as aromatic carbohydrates and (poly) chlorinated aliphatic and aromatic hydrocarbons. Although Phase

I reactions involve a wide range of chemical transformations from hydrolysis to reduction, the most common Phase I reactions are oxidative processes that involve cytochrome P450 enzymes (Doty, 2008).

These enzymes support the oxidative, peroxidative and reductive metabolism of both endogenous and xenobiotic substrates. They comprise of a superfamily of heme-thiolate proteins present in every class of organism. Cytochrome P450 enzymes are characterized with high diversity of reactions that they catalyze and a high range of their chemically divergent substrates. Increasing emphasis on functional genomic approaches to P450 research recently has greatly advanced our understanding of cytochrome P450-mediated reactions in plants. Transgenic plants with tailored Phase I enzymatic activities may play major roles in the removal of environmentally stable organic pollutants from contaminated fields (Doty, 2008).

These findings are in agreement with a recent study conducted on mice liver microsomes in which pretreatment of mice with pyrazole, which induces CYP2E1, enhanced lipid peroxidation due to CH, whereas addition of a general P450 inhibitor reduced CH-induced lipid peroxidation. This suggests that metabolism of CH to TCOH and TCA is catalyzed primarily by CYP2E1 (Doty *et al.,* 2007).

Another study also found that hybrid cottonwood trees are commonly used for phytoremediation applications because of their water uptake potential and the ability to degrade TCE. The cottonwood and sycamore trees exhibit the ability to degrade TCE into TCEOH, as well as high trichloroacetic acid (TCAA). Plant's ability to degrade TCE differs with plants species and certain native southeastern plants are capable of degrading TCE and may be used for phytoremediation (Doty *et al.,* 2003).

3.3 Potassium, Manganese, Copper and Lead accumulation in transgenic plants

3.3.1 Potassium accumulation in transgenic plants

Results displayed in Figure 3.19 indicate that an increase in the accumulation of K in transgenic *S. grandiflora* reached 13, 37 and 55% as compared with wild type plants in all three doses (200, 250 and 300ppm) respectively. When soil samples were analysed, a reduction in K level reached 37, 50, and 13% for the three used doses compared with control samples.

A. thaliana transgenic lines showed increased K accumulations up to 33, 54 and 13% compared with wild type plants in all doses (Figure 3.20). A decrease in K concentrations reached 73, 20 and 7% in soils treated with three different doses of K and planted with *A. thaliana* transgenic plants.

Transgenic *S. grandiflora* and *A. thaliana* lines, expressing the cyp450 2E1 exhibited normal growth under high levels of metals. This could be due to the absorption of K ion from the vicinity of the roots then moved to other plant parts at times and stages when plant growth is not that sensitive to high level of potassium. Salt-induced depolarization of the root plasma membrane may activate outward rectifying potassium channel, enabling the diffusion of Na^+ into the cells down its electrochemical gradient (Schachtman *et al.,* 1991).

These results are supported by Laurie *et al.* (2002) and Berthomieu *et al.* (2003) who reported that transgenic wheat lines, expressing the HKT1, exhibited enhanced growth under salinity, due to reduced Na^+/K^+ ratio when compared with control plants.

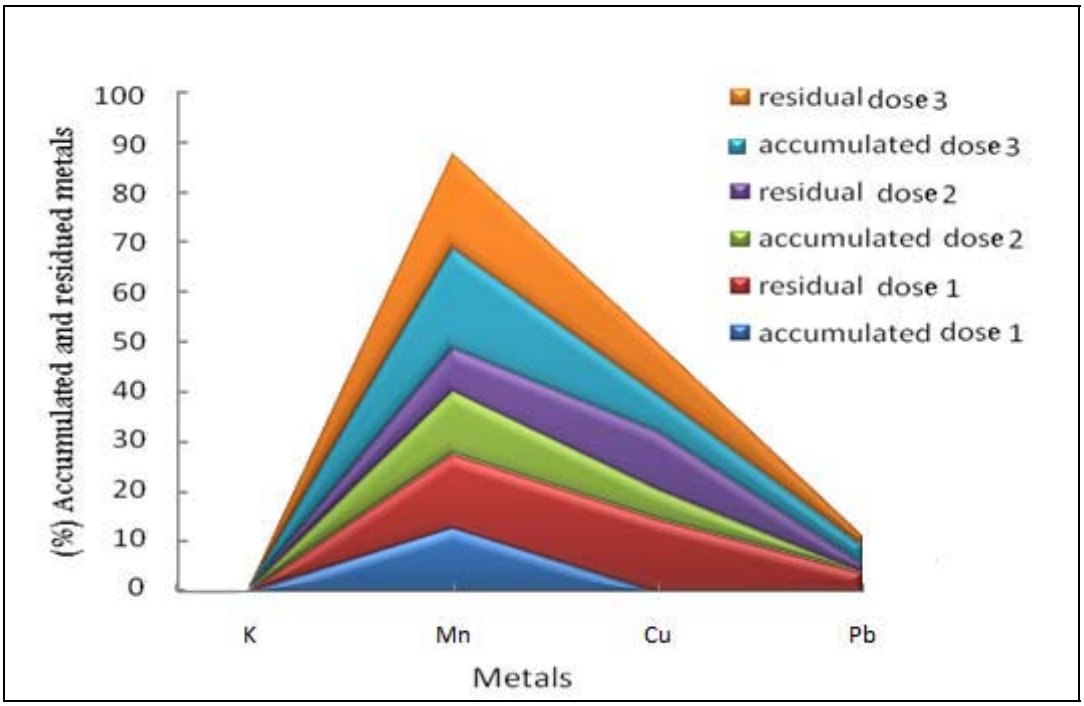

Figure (3.19): Metal accumulation in *S. grandiflora* transgenic plants and residual concentrations in soil after 12 days of planting.

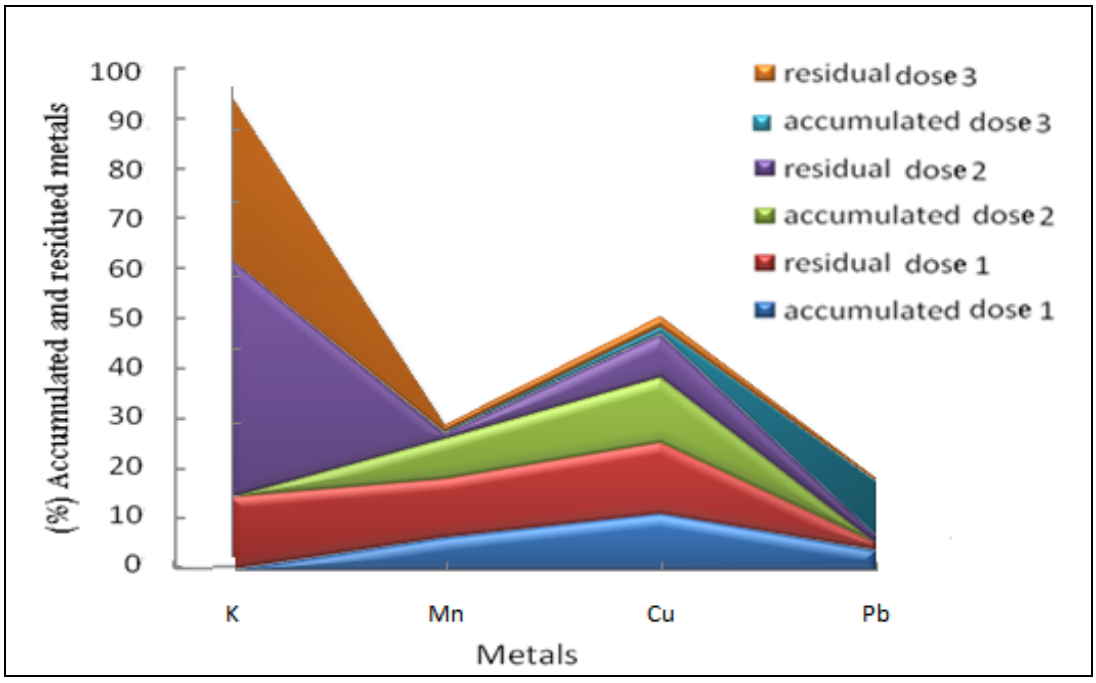

Figure (3.20): Metal accumulation in *A. thaliana* transgenic plants and residual concentrations in after 12 days of planting.

3.3.2 Manganese accumulation in transgenic plants

Results (Figure 3.19) show increased accumulation of Mn in transgenic *S. grandiflora* reached 43, 54, and 43% as compared with wild type plants in the three doses. When soil samples were tested, a decrease in Mn level (10, 59, and 31%) was recorded compared with control samples.

Similarly, *A. thaliana* transgenic lines showed increased accumulation recording 15, 68 and 17% for the three doses respectively (Figure 3.20). While a decrease in Mn concentration occurred (47, 21 and 32%) in all doses of soil planted with *A. thaliana* transgenic plants.

The reduction in Mn may be due to a shift of manganese to accessible formula in the transgenic plant roots to make it not toxic to plant (Tisdale *et al*., 1997).

3.3.3 Copper accumulation in transgenic plants

Figure 3.19 exhibited an increased accumulation of Cu^{2+} in transgenic *S. grandiflora* reached 6, 52, and 42% as compared with wild type plants in all three doses. Soil analysis revealed, a decrease in Cu concentration reached 27, 18 and 55% compared with control samples.

A. thaliana transgenic lines accumulated high percentage of Cu reached 28, 28 and 44% compared with wild type plants in all three doses respectively. On the other hand, a decrease of Cu (31, 31 and 38%) was recorded in all doses added to the soil and planted with *A. thaliana* transgenic plants (Figure 3.20).

Transgenic *S. grandiflora* and *A. thaliana* lines, expressing the cyp450 2E1 exhibited normal growth under high concentrations of Cu^{2+}.

Copper is an important redox component directly related to the electron transfer reactions in photosynthesis, respiration, lignification of cell walls and detoxification of superoxide radicals (Fox and Guerinot, 1998). Amino acids, nicotianamine and histidine, are involved in chelation of copper ions in the xylem sap (Liao *et al*., 2000).

3.3.4 Lead accumulation in transgenic plants

Figure 3.19 displays an increased accumulations of Pb in transgenic *S. grandiflora* reached 58, 1.7 and 25% as compared with wild type plants in all three doses (30, 40 and 50 ppm). The figure also shows a decline in Pb reached 18, 42, and 40% in all soils treated with the three different concentrations and planted with transgenic *S. grandiflora* compared with control samples.

A. thaliana transgenic lines showed an increased percentage in Lead reached 100% for the first dose (30 ppm) as shown in figure 3.20. While a decrease in element concentration occurred at percentage 28, 28 and 44% in all doses of soil planted with *A. thaliana* transgenic plant.

Transgenic *S. grandiflora* and *A. thaliana* lines, expressing the cyp450 2E1 exhibited normal growth under high concentration of Lead. Transgenic *A. thaliana* plants grown in mining soils containing high levels of Pb accumulated twice the level of this heavy metal than wild type plants (Lee *et al.,* 2003).

3.3.5 Metal effects on plant fresh and dry weight

S. grandiflora and *A. thaliana* transgenic plants grown on soils contaminated with metals K (200, 250 or 300ppm), Mn (75, 100 or 125ppm), Cu (75, 100 or 125ppm) and Pb (30, 40 or 50ppm) recorded higher dry weight than wild type plants 12 days after soil contamination (Table 3.11).

Table (3.11): Effect of Potassium, Manganese, Copper and Lead mixture at three doses (ppm) on plants fresh and dry weight (g) after 12 days of soil contamination.

Plants	Dose 1		Dose 2		Dose 3	
	Fresh weight (g)	Dry weight (g)	Fresh weight (g)	Dry weight (g)	Fresh weight (g)	Dry weight (g)
Transgenic *A. thaliana*	1.369	0.152	1.566	0.198	2.509	0.138
Wild type *A. thaliana*	0.490	0.052	0.078	0.022	0.346	0.109
Transgenic *S. grandiflora*	3.734	0.188	3.527	0.165	4.420	0.196
Wild type *S. grandiflora*	0.117	0.081	0.446	0.097	0.548	0.095

Transgenic *S. grandiflora* plants grown on metal contaminated soils had much higher fresh weight (4.42g) than wild type plants (0.54g).

Similarly, higher dry weight (0.196g) was obtained in transgenic *S. grandiflora* compared with wild plants (0.09g). Transgenic *A. thaliana* plants grown on metal contaminated soils had much higher fresh weight (2.50g) than wild type plants (0.346g).

Similarly, higher dry weight of 0.198g was obtained in transgenic *A. thaliana* compared with wild plants (0.022g). *A. thaliana* plants had lower

fresh weight than *S. grandiflora* plants, which could be due to the fact that *A. thaliana* plants had shorter life cycle and smaller size.

Kozhevnikova *et al.* (2006) stated that toxic effects of metals were assessed from inhibition of the primary root length increment as compared to the roots grown in water. Metal accumulation was found both in the meristem and in the root cap. Pb and Sr were revealed primarily in the cell walls, metal content in all root tissues was decreased, and their toxic effect on root growth was ameliorated.

3.4 Effect of contaminated soil with Calcium, Iron, zinc and Brom

3.4.1 Calcium accumulation in transgenic plants

Figure 3.21 shows increased Ca accumulations in transgenic *S. grandiflora* reached 45, 10, and 45% as compared with wild type plants at the three sampling times. Moreover, it shows a decline reached 10, 59, and 31% in soil samples at different sampling times planted with transgenic *S. grandiflora* compared with wild type plants.

A. thaliana transgenic lines show increased accumulations reached 29, 64 and 7% as compared with wild type plants at the three sampling times respectively. While a reduction reached 37, 39 and 24% in contaminated soils planted with *A. thaliana* transgenic plant after 5, 10 and 15 days of planting respectively (Figure 3.22). Transgenic *S. grandiflora* and *A. thaliana* lines, expressing the cyp450 2E1 exhibited normal growth under high concentrations of metals due to a reduced toxicity of Calcium which has different binding forms and compartmentalize within the cell.

Results were in agreement with Evans *et al.* (1991); Tester and Davenport (2003) who reported that the increase in Ca accumulation could be explained since Ca in an essential plant nutrient and is acquired from the soil solution by the root system and translocated to the shoot via the xylem.

3.4.2 Iron accumulation in transgenic plants

Results show increased accumulation of Fe^{+2} in transgenic *S. grandiflora* reached 17, 75, and 8% as compared with wild type plants at the three sampling times (Figure 3.21).

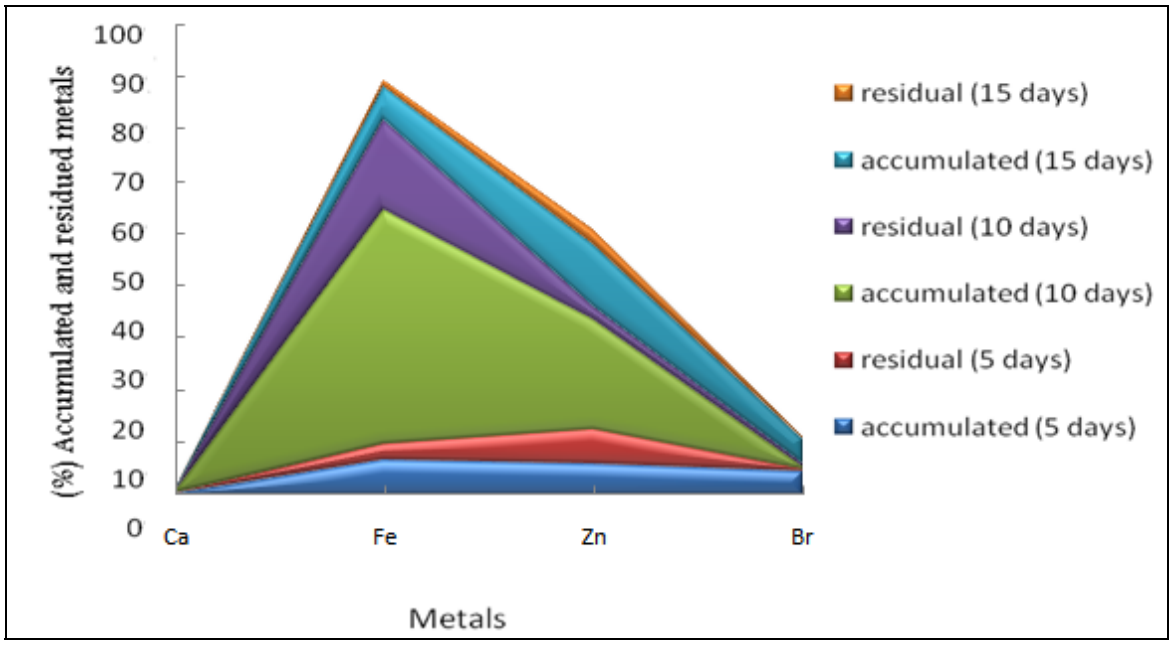

Figure (3.21): Metal accumulation in *S. grandiflora* transgenic plants after 5, 10 and 15 days of planting and in soils planted with *S. grandiflora* transgenic plants.

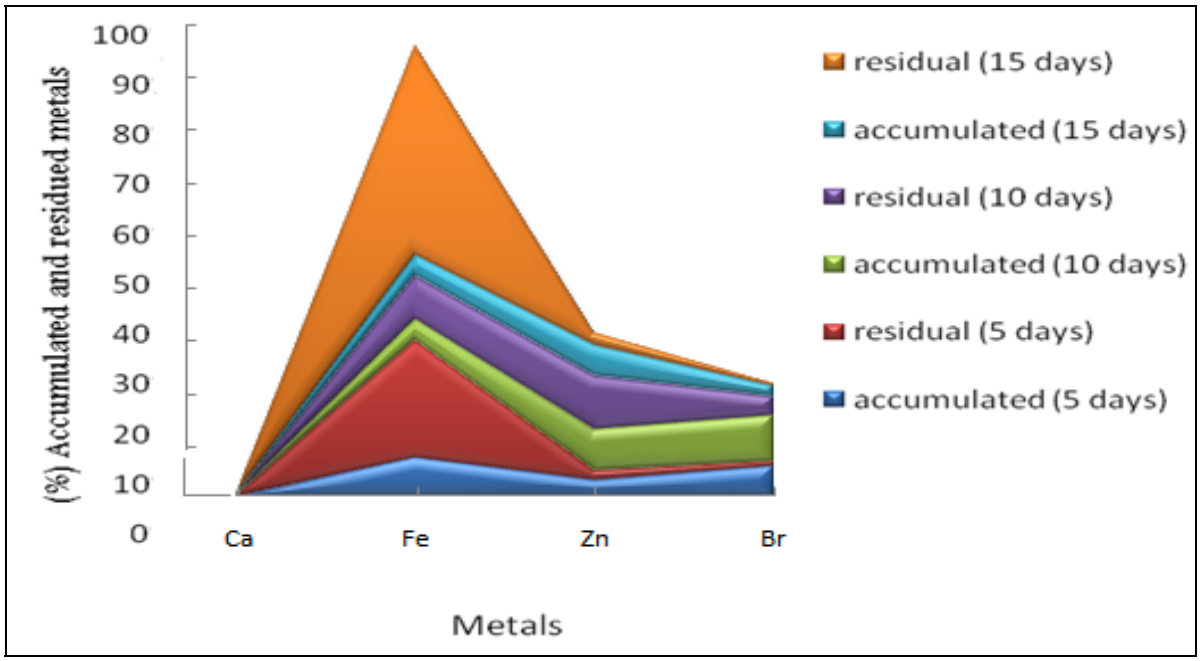

Figure (3.22): Metal accumulation in *A. thaliana* transgenic plants after 5, 10 and 15 days of planting and in soils planted with *A. thaliana* transgenic plants.

While a decrease reached 12, 76, and 12% of Iron was obtained in soil samples at different times planted with transgenic *S. grandiflora* compared with soils planted with wild type plants.

A. thaliana transgenic lines showed increased accumulation of Iron reached 57, 42, 1% compared with wild type plants at the three times of sampling respectively. While decreases in Iron concentration were 58, 14 and 28% in soil planted with *A. thaliana* transgenic plants after 5, 10 and 15 days of planting (Figure 3.22).

Iron is a component of heme proteins, cytochromes, cytochrome oxidase, catalase, peroxidase, leghemoglobin and nonheme proteins like ferredoxin and lipoxygenase and thus it is essential for the biosynthesis of chlorophyll (Pushnik and Miller, 1989). The role of Iron is hypothesized by Eide *et al.* (1996) and Schaaf *et al*. (2004) who reported that ZmYS1 encodes a proton-coupled broad-range metal-phytosiderophore transporter that additionally transports Fe and Ni-nicotianamine. These biochemical properties indicate a novel role for YS1 transporters for heavy metal homeostasis in plants.

3.4.3 Zinc accumulation in transgenic plants

Results displayed in figure 3.21 show increased accumulation of Zn in transgenic *S. grandiflora* recording 43, 54, and 43% as compared with wild type plants after 5, 10 and 15 days of planting respectively. The figure also shows a decrease reached 7, 13 and 80% of Zinc in all soils samples at different times and planted with transgenic *S. grandiflora* compared with that planted with wild type plants. *A. thaliana* transgenic lines showed increased accumulation of Zinc reached 28, 28 and 44% compared with wild type plants at the three sampling times respectively. While decreases occurred in

Zinc accumulation recording 47, 21 and 32% in soils planted with *A. thaliana* transgenic plant (Figure 3.22). Zinc is an important component for plant growth and development but the amount needed by the plant is very small not exceeding 0.002% (Nelson *et al.,* 1983). It is suggested by Delhaize *et al.* (2003) that HMA4 (transporter protein) was responsible for the transport of zinc in leaves of *A. thaliana*.

3.4.4 Brom accumulation in transgenic plants

Results show increased accumulation of Br in transgenic *S. grandiflora* reached 51, 6, and 43% as compared with wild type plants at all selected times (Figure 3.21). However, a decrease reached 27, 45, and 28% of Brom was recorded in soils treated at different times and planted with transgenic *S. grandiflora* compared with soils planted with wild type plants.

A. thaliana transgenic lines exhibited increased accumulation of Brom reached 15, 68 and 17% as compared with wild type plants at all sampling times respectively. While reductions reached 18, 17 and 11% in Brom in soils planted with *A. thaliana* transgenic plants (Figure 3.22).

High levels of Brom lead to toxicity and inhibition of plant growth in wild type plants, while transgenic plants with cytochrome p450 2E1 are capable to accumulate Brom in cell tissue. Schaaf *et al*. (2004) reported that ZmYS1 encodes a proton-coupled broad-range metal-phytosiderophore transporter. These biochemical properties indicate a novel role for YS1 transporters for heavy metal homeostasis in plants.

3.5 Effect of radionuclide Uranium, Cesium and Strontium

3.5.1 Germination of *A. thaliana* seeds in the presence of Uranium presence

In order to evaluate the effect of Uranium (^{238}U) on wild type *A. thaliana*, seeds were sown on a medium containing different ^{238}U concentrations (0, 25 or 50 mM of ^{238}U). After seven days, germination started in all treatments reaching 87.1% in the control (0mM ^{238}U) and declined with increasing ^{238}U level over time course. At the end of day 15, germination percentages reached 99.3, 65.7 and 29.3% for the ^{238}U concentrations (0, 25 and 50mM) respectively (Figure 3.23).

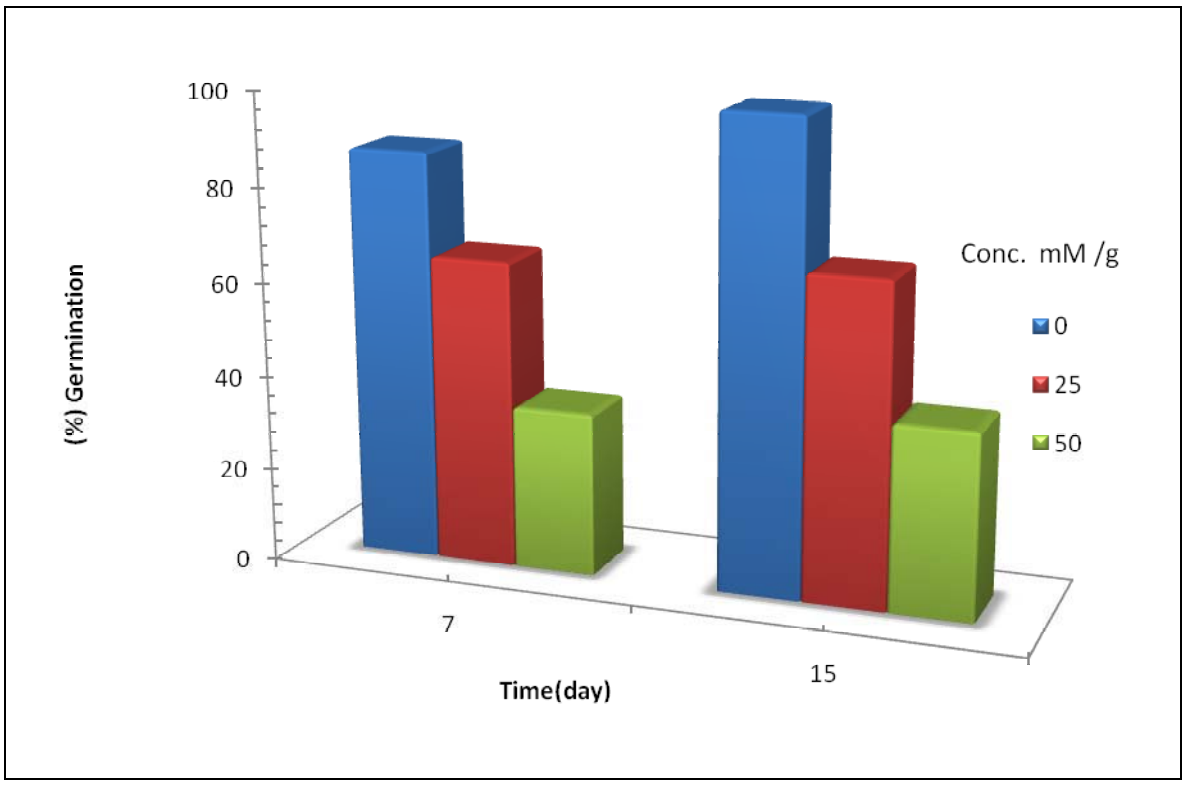

Figure (3.23): Germination percentages of *A. thaliana* seeds at 7 and 15 days after sowing on different ^{238}U concentrations (0, 25 and 50mM).

3.5.2 Increased accumulation of Uranium in *A. thaliana*

A. thaliana transgenic lines showed increased translocation of ^{238}U compared with non transgenic plants (control). F1 and F2 trangenic plants were able to translocate 90% and 80% of ^{238}U respectively (Figure 3.24).

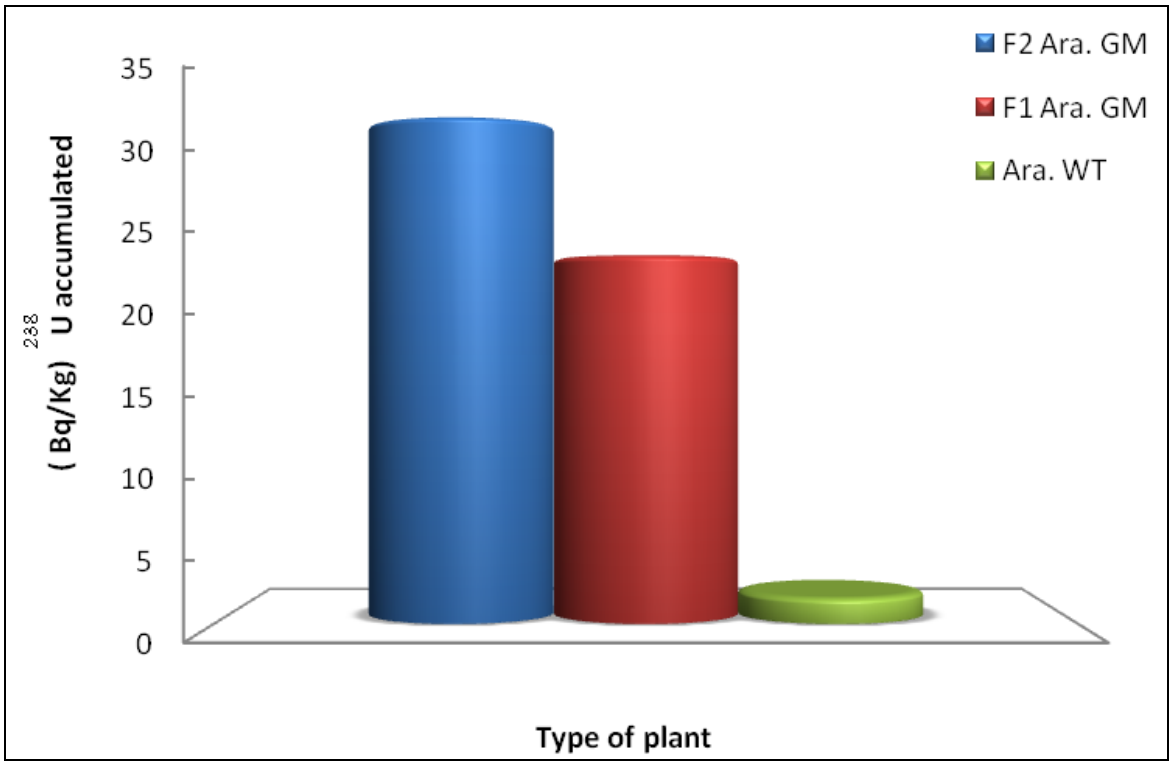

Figure (3.24): Concentration of ^{238}U in transgenic *A. thaliana* F1 (F1 Ara.GM) and F2 (F2 Ara.GM) progeny and in wild type plants (Ara.Wt) after 12 days of planting.

3.5.3 Decreased concentration of Uranium in soil planted with *A. thaliana*

^{238}**U** was decreased by 50 and 60% in soils planted with *A. thaliana* F1 and F2 plants respectively compared with soils planted with wild type plants (Figure 3.25).

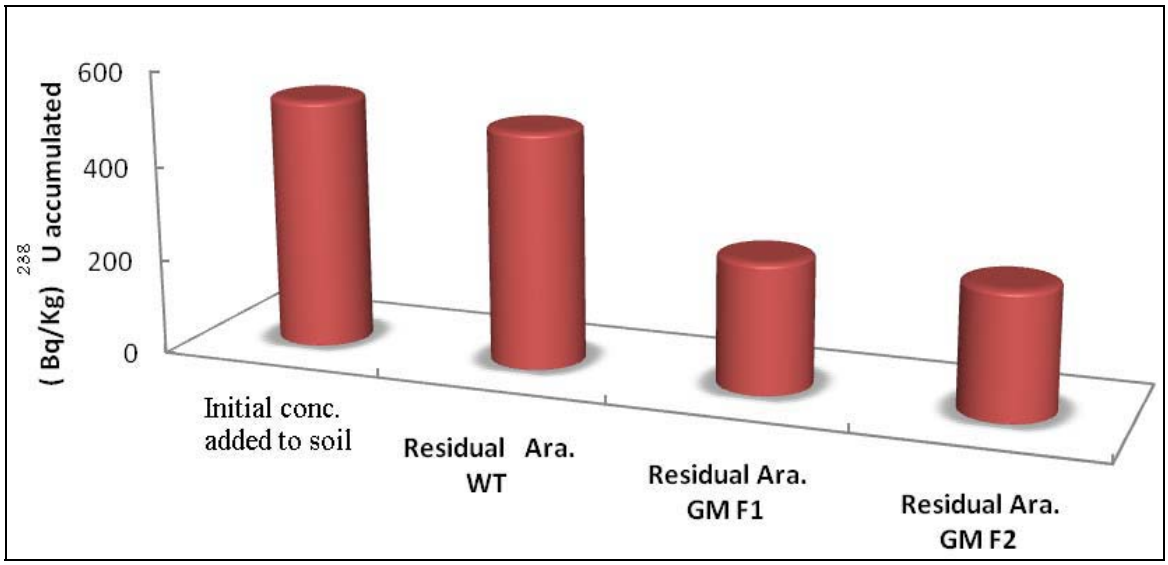

Figure (3.25): Concentration of ^{238}**U** in soil planted with transgenic *A. thaliana* F1 and F2 progeny and wild type compared with the initial concentration added to the soil after 12 days of planting.

Cytotoxicity of ^{238}**U** was prevented by ROS (reactive oxygen species) scavengers, antioxidants, and glutamine (ATP generator). Hepatocyte dichlorofluorescein oxidation was inhibited by mannitol (a hydroxyl radical scavenger) or butylated hydroxyanisole and butylated hydroxytoluene (antioxidants). Glutathione depleted hepatocytes were resistant to ^{238}**U** (VI) toxicity and much less dichlorofluorescein oxidation occurred. Reduction of U (VI) by glutathione or cysteine *in vitro* was also accompanied by oxygen uptake and was inhibited by Ca (II), a U (IV) or U (VI) reduction inhibitor. U (VI)-induced cytotoxicity and ROS formation was also inhibited by Ca (II), which suggests that U (IV) and U (VI) mediate ROS formation in isolated

hepatocytes. The U (VI) reductive mechanism required for toxicity has not been investigated (Jalal *et al*., 2006). Cytotoxicity was also prevented by cytochrome P450 inhibitors, particularly CYP2E1 inhibitors, but not inhibitors of DT diaphorase or glutathione reductase. This suggests that P450 reductase and reduced cytochrome P450 contribute to U (VI) reduction to U (IV). In conclusion, U (VI) cytotoxicity is associated with mitochondrial/lysosomal toxicity by the reduced biological metabolites and ROS (Dushenkov, 2003).

3.5.4 Increased translocation of ^{90}Strontium in *A. thaliana* and *S. grandiflora*

A. thaliana and *S. grandiflora* plants were strongly expressed the enzyme (P450) as determined by Western blot analysis after using *A. tumefaciens*-mediated transformation. Results showed increased accumulation of ^{90}Sr in transgenic *S. grandiflora* reached 60% compared with wild type plants (Figure 3.26). *A. thaliana* transgenic lines showed increased accumulation 70% compared with wild type plants which reached 65%. A decrease in element concentration in soils planted with transgenic *A. thaliana* and *S. grandiflora* recording (50%) compared with wild type plants (Figure 3.27).

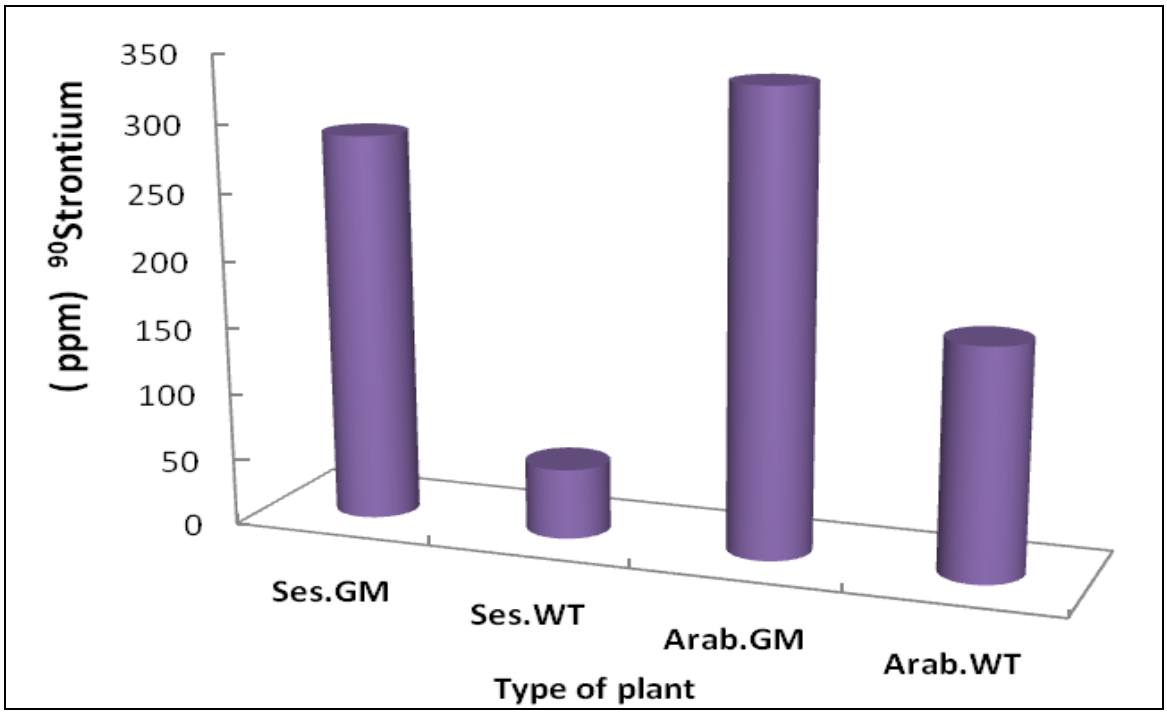

Figure (3.26): Concentration of ^{90}Sr in *A. thaliana* and *S. grandiflora* transgenic and wild type plants (control) after 7 days.

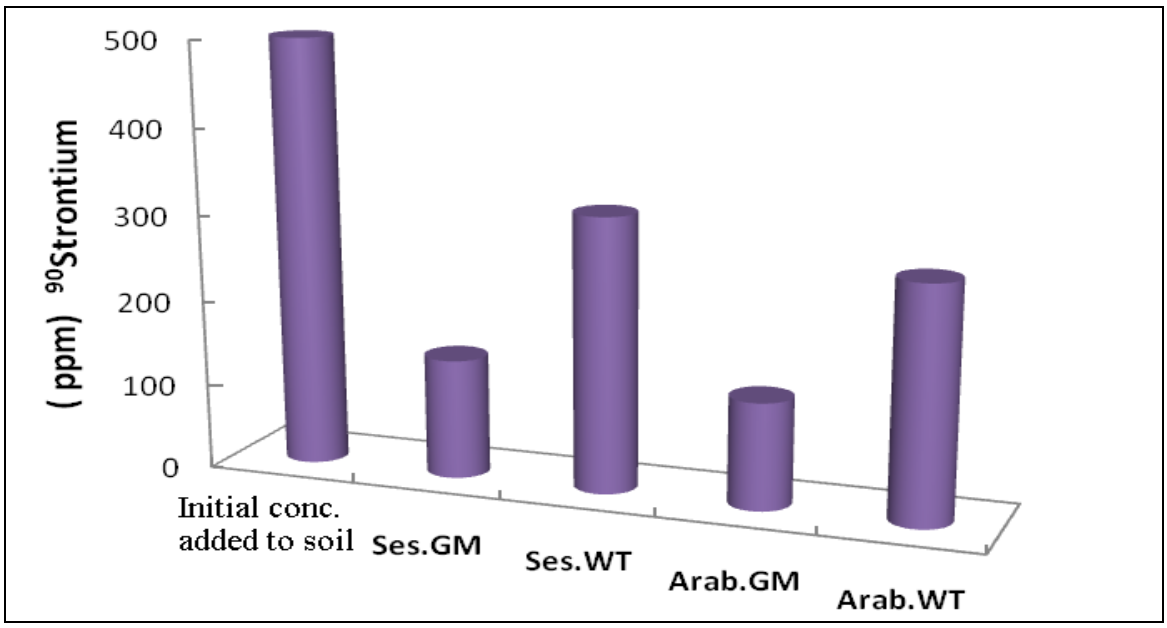

Figure (3.27): Concentration of ^{90}Sr in soils planted with transgenic *A. thaliana* and *S. grandiflora* plants and wild type plants (control) after 7 days.

It is clear from the above presented data that *A. thaliana* and *S. grandiflora* transgenic plants have developed mechanisms that inactivate or chelate the metal ion upon its entry into the plant cytosol. This system prevents the metal from inactivating or activating structural proteins, whilst at the same time allowing elements essential for the plant's metabolic function to be taken up and transformed into forms that are tolerable to the plant.

These results are supported by Dushenkov (2003) who reported that the toxic effects of metals are assessed from inhibition of the primary root length increment following 24 hrs and 48 hrs incubations as compared to the roots grown in water or in 3mM $Ca(NO_3)_2$ solution. Metal localization in the root apex tissues following 24 hrs and 48 hrs incubations was determined using histochemical techniques. In the absence of Ca $(NO_3)_2$, metals were found in both the meristem and in the root cap. Pb and ^{90}Sr were revealed primarily in the cell walls. It was suggested that inhibition of divisions of the root cap upper layer cells and a decrease in the sloughing off its cells can stimulate the quiescent center cell divisions.

3.5.5 Increased translocation of ^{137}Cesium in *A. thaliana* and *S. grandiflora*

Our data show increased translocation of ^{137}Cs around 60% in transgenic *S. grandiflora* compared with wild type plants. *A. thaliana* transgenic lines showed higher translocation 70% compared with wild type plants and *S. grandiflora* transgenic (Figures 3.28; 3.29; 3.30; 3.31 and 3.32).

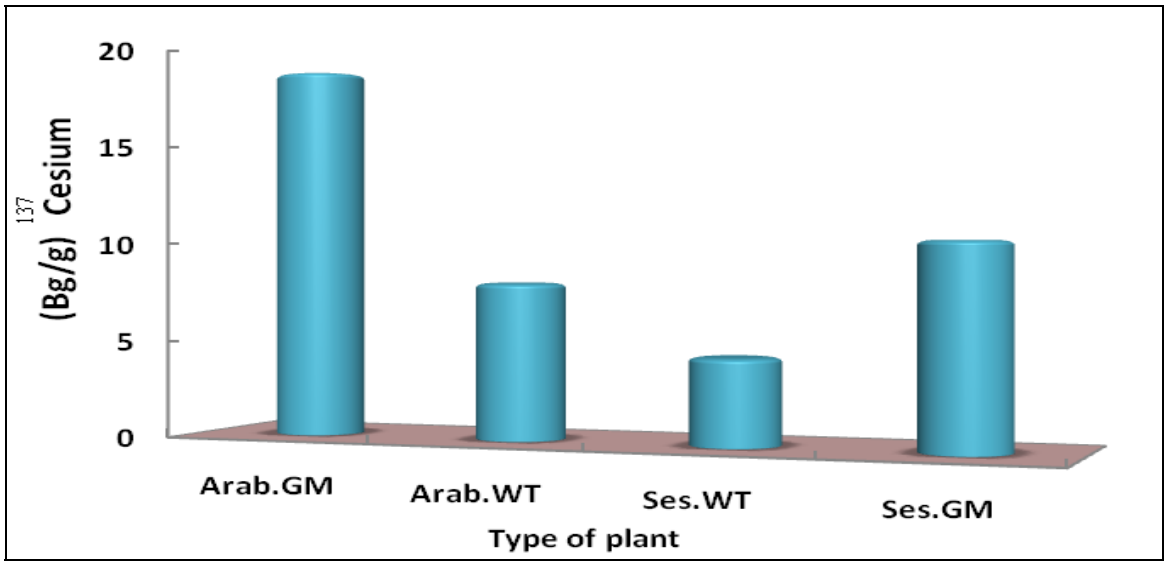

Figure (3.28): Concentration of ^{137}Cs in transgenic *A. thaliana* and *S. grandiflora* plants and wild type plants (control) after 7 days.

Figure 3.29 shows a 40% decrease of ^{137}Cs concentration in soils planted with transgenic *S. grandiflora* compared with wild type plants. A decrease of 30% in ^{137}Cs concentration was seen in soil planted with transgenic *A. thaliana* plants.

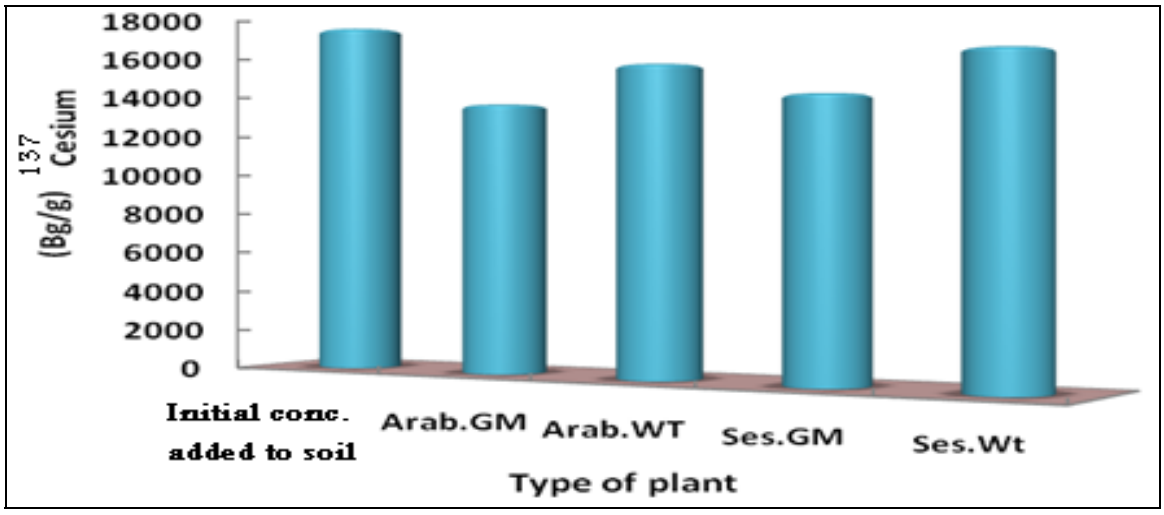

Figure (3.29): Concentration of ^{137}Cs in soils planted with transgenic *A. thaliana* and *S. grandiflora* plants and wild type plants (control) after 7 days.

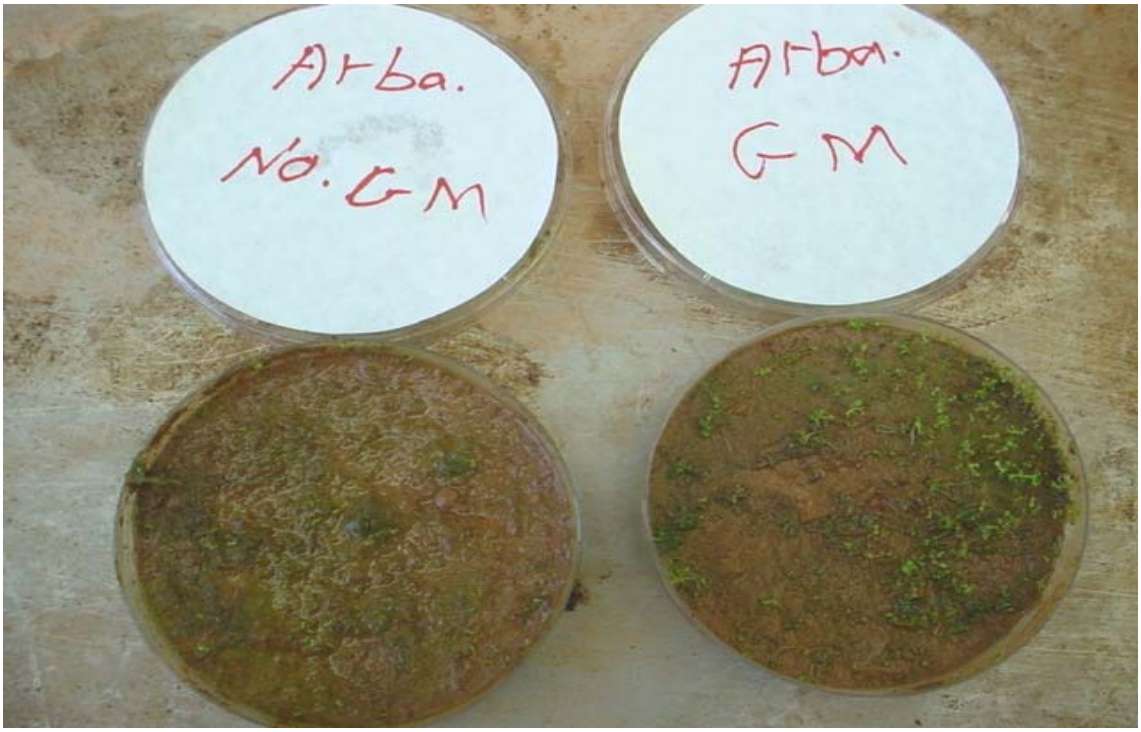

Figure (3.30): Transgenic and wild type (control) *A. thaliana* plants grown on soil contaminated with 17600 Bq/g ^{137}Cs.

Figure (3.31): Transgenic *S. grandiflora* plants grown on soil contaminated with 17600 Bq/g ^{137}Cs.

Figure (3.32) *S. grandiflora* wild type (control) grown on soil contaminated with 17600 Bq/g ^{137}Cs.

Cesium accumulation in leaves may disturb their basic physiological functions. The first observed reaction in cesium-treated plants was a decreased stomatal opening. The control function of stomata in respect to photosynthetic is CO_2 assimilation and transpiration that were found to be modified by the presence of cesium. Decreased stomatal opening limits transpiration and the uptake of water by roots, but photosynthetic CO_2 assimilation is not changing during short-term exposure to CsCl. Stomatal closure in the presence of cesium may be a result of a decrease in osmotic potential due to ^{137}Cs accumulation in the vacuoles (Chen *et al.,* 2003).

Furthermore, Zhu *et al.* (2002) reported that ^{137}Cs possibly inhibits the channels responsible for K transport into the guard cells, which might lead to impaired stomatal opening and restricts production of assimilates important to dry mass accumulation in cress. Although photosynthesis and transpiration were affected by cesium, photosynthetic water utilization efficiency was rather stable. The lowering of chlorophyll fluorescence seems to be a secondary effect of a disturbed biosynthesis. It also lists the associated plant species that have accumulated radionuclides due to their increased bioavailability. In a greenhouse study, ammonium nitrate and ammonium chloride increased the uptake of ^{137}Cs into cabbage, Tepary beans, Indian mustard (*Brassica juncea*), and reed canary grass.

Moreover, rhizofiltration, a way of removing radionuclides from contaminated soils, is most effective when plant roots have large densities and radionuclides are available for plant uptake (Entry *et al*., 1997).

Two plants that have proven the most successful at rhizofiltration are sunflower (*Helianthus annuus*) and water hyacinth (*Eichornia crassippes*). Both have been found to accumulate significant percentages of radionuclides (^{137}Cs, U, ^{90}Sr) within a few hrs to a few days (Negri and Hinchman, 2000).

Conclusions and Recommendations

Conclusions

1. Cytochrome P450 2E1 is a rabbit enzyme with broad specificity for removal of environmental pollutants including TCE, DDT, heavy metals and radionuclides. In a proof-of-concept project, introduction of a rabbit CYP2E1 gene into *Sesbania* and *Arabidopsis* plants resulted in plants with increased metabolism of these pollutants.
2. First transgenic *S. grandiflora* and *A. thaliana* with *CYP2E1* were developed for enhanced phytoremediation capabilities for removing and degrading several of the most widespread and dangerous pollutants from the environment.
3. Transgenic *S. grandiflora* and *A. thaliana* plants showed an increased average in DDT metabolism that was nearly 85-fold greater than average seen in the control plant.
4. Transgenic *A. thaliana* and *S. grandiflora* plants showed an increased TCE metabolism that was nearly 75 fold greater than that found in controls.
5. The ability of transgenic *S. grandiflora* and *A. thaliana* plants in remediation of inorganic pollutants (Mn, Fe, Cu, Zn, Br, Pb) efficiently achieving 54, 52, 51, 54, 58, and 51% compared with non-transgenic plants.
6. *A. thaliana* transgenic lines showed an increased ^{238}U translocation recording 90% and 80% for the F1 and F2 progenies respectively compared with wild type plants.
7. *A. thaliana* transgenic lines showed higher (^{90}Sr, ^{137}Cs) translocation (70%) compared with wild type plants and transgenic *S. grandiflora.*

Recommendations

The following points can be recommended for further studies

1. Studying the pathways of pollutants in plant tissues to achieve better understanding for the mechanism involved.
2. Introduction of the gene CYP2E1 to other plant species to enhance environmental clean up.
3. Studying P450 2E1 heritability and stability in *Sesbania grandiflora* progeny.
4. Studying the effects of possible toxicity for grazing animals by CYP2E1 since the plant (*Sesbania grandiflora*) is used as a forage crop.

References

References:

Abbot, R. J. and Gomes, M. F. (1989). Population genetic structure and outcrossing rate of *Arabidopsis thaliana* (L.) Hennh.headerity , 62:411-418.

Agarwal, P. K.; Akhtar, M. and Anwar, M. (1997). Basmati rice progress, plant regeneration from maintained calli. Oryza., 43: 10-15.

Aken, B.; Peres, C. M.; Doty, S. L.; Yoon, J. M. and Schnoor, J. L. (2004). Methylobacterium oliven ane-utilizing bacterium isolated from poplar trees. (*Populus deltoids X Populus nigra* DN34). Intern. J. of Sys. and Evo. Microb., 54: 1191–1196.

Al-Daoude, A. (2003). RIN13 is a positive regulator of the plant disease. M.Sc. thesis, Univ. of Imperial, London, UK.

Al-Daoude, A.; Zabala, M; Ko, J; T. Z.; Jong-Hyun, K. and Grant, M. (2005). RIN13 is a positive regulator of the plant disease resistance protein RPM1. The Plant Cell, 17: 1016–1028.

Allavena, H. and Sarp, T. (1981). *Agrobacterium tumefaciens* transformation of japonica and indica varieties of rice. Planta, 199: 612-617.

Anderson, T. A. (1996). Comparative fate of [14C] trichloroethylene in the root zone of plants from a former solvent disposal site. Environ. Toxicol. Chem., 12: 2041–2047.

Ashis, T. Henry, H. and Blakeney, E. (2007). Varietal differences in plant regeneration from root callus tissue of rice. J. P. Breed., 34: 187-196.

Atlas, R. M.; Brown, A. E. and Parks, L. C. (1995). Laboratory Manual of Experimental Microbiology (1st ed.). Mosby, Inc. Missouri, USA.

Baily, S.; Baron, E. and Finegold, S. (1999). Diagnostic Microbiology 8th ed. The Mosby, Co. London.

Baker, A. J. M. (1999). Metal hyperaccumulator plants: A review of the biological resource for possible exploitation in the phytoremediation of metal-polluted soils. In: Phytoremediation of Contaminated Soil and Water, 6: 85–107.

Banerjee, S.; Shang, Q. T.; Wilson, A. M.; Moore, A. L.; Strand, S. E.; Gordon, M. P. and Doty, S. L. (2002). Expression of active mammalian P450 2E1 in hairy root cultures. Biotech. Bioeng., 77: 462–466.

Banuelos, G.; Terry, N.; LeDuc, D. L.; Pilon-Smits, E. A. and Mackey, B. (2005). Field trial of transgenic Indian mustard plants shows enhanced phytoremediation of selenium-contaminated sediment. Environ. Sci. and Techn., 39: 1771–1777.

Barac, T.; Taghavi, S.; Borremans, B.; Provoost, A.; Oeyen, L.; Colpaert, J. V.; Vangronsveld, J. and van, L. D. (2004). Engineered endophytic bacteria improve phytoremediation of water-soluble, volatile, organic pollutants. Nat. Biotech., 22: 583–588.

Bechtold, N., Elis, J. and Pelletetier, G. (1993). In planta *Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana*. C. R. Acad. Sci. Paris, life Sci., 316: 1194-1199.

Berenbaum, M. R. and Zangerl, A. R. (1996). Physico-chemical diversity: adaptation or random variation. Recent Advances in Phytoch., 30: 1–12.

Berthomieu, P.; Conejero, G.; Nublat, A.; Brackenbury, W. J.; Lambert, C. and Savio, C. (2003). Functional analysis of AtHKT1 in Arabidopsis shows that Na^+ recirculation by the phloem is crucial for salt tolerance. Embo J., 22: 2004-2014.

Bewley, F. and Black, D. (1994). Enhanced somatic embryogenesis in *Sorghum bicolor* L. from shoot tip culture *in vitro*. Cell Dev. Biol., 24: 65-70.

Bhadra, R.; Wayment, D. G.; Williams, R. K.; Barman, S. N.; Stone, M. B; Hughes, J. B. and Shanks, J. V. (2001). Studies on plant-mediated fate of the explosives RDX and HMX. Chemosphere, 44: 1259–1264.

Birnboim, H.C. and Doly J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res., 7: 1513–1523.

Bizily, S. P.; Rugh, C. L. and Meagher, R. B. (2000). Phytodetoxification of hazardous organomercurials by genetically engineered plants. Nature Biotech., 18: 213–217.

Boyajian, G. E. and Carreira, L. H. (1997). Phytoremediation: Aclean transition from laboratory to marketplace. Nature Biotech., 15: 127–128.

Brown, S. L.; Chaney, R.; Angle, J. and Baker, A. J.; (1995). Zinc and cadmium uptake by hyperaccumulator *Thlaspi caerulescens* grown in nutrient solution. Soil Sci. Soci. of America J., 59: 125–132.

Bruning, T. and Bolt, H. (2000). Renal toxicity and carcinogenicity of trichloroethylene: key results, mechanisms and controversies. Critical Reviews in Toxic., 30: 253–285.

Bumpus, J. A. and Aust, S. D. (1987). Biodegradation of DDT [1,1 1-Trichloro-2,2-Bis(4-Chlorophenyl)Ethane] by the white rot fungus *Phanerochaete chrysosporium*. Applied and Environ. Micro., 53: 2000-2008.

Burken, J. and Schnoor, J. L. (1998). Predictive relationships for uptake of organic contaminants by hybrid poplar trees. Environ. Sci. and Techn.., 32: 3379–3385.

Campanella, B. (2000). Impact of root exudates on bioavailability of hydrophobic organic pollutants. Inter Cost workshop on Bioremediation. Cost Action, 831: 15–18.

Carreira, L. H. and Wofe, N. L. (1992). Isolation of a sediment nitroreductase, antibody production, and identification of possible plant sources. Plant Biotech. J., 6: 67-75.

Chaney, R. L. (1988). Plants can utilize iron from Fe-N-N-DI-(2-hydroxybenzoyl)-ethylene, diamine–N-N-Diacetic acid, a ferric chelate with 106 greater formation constant than Fe- EDDHA. J. Plant Nutrition, 11: 1033–1050.

Chappell, J. (1998). Phytoremediation of TCE in groundwater using *Populus*. Prepared for US Environ. Protec. Agency.

Char, W. P. (1983). A revision of the Hawaiian species of *Sesbania* (Leguminosae). M.Sc. thesis, Univ. of Hawaii, Hawaii, USA.

Chen, C. D.; Rogers, S.; Kemper, B. and Szczesna-Skorupa, E. (2003). Cs accumulation in vacuoles plant. Proc. Nature, 81: 8500–8505.

Chiu,T.; Zhimou, W.; Sanjeewa, G.; and Mary, A. (2008) Comparative molecular modeling of Anopheles gambiae CYP6Z1, a mosquito P450 capable of metabolizing DDT. Proc. Natl. Acad. Sci., 105: 8855–8860.

Cherian, S. and Oliveira, M. M. (2005). Transgenic plants in phytoremediation: Recent advances in Phytoch., 5: 33-37.

Cho, M.; Yano, H. and Okamoto, (2004). Improvement of shoot regeneration from sculelium-derived callus in rice. Korean J. Crop. Sci., 49: 52-60.

Coleman, J. O. (1992). Detoxification of xenobiotics by plants: Chemical modification and vacuolar compartmentation. Trends in Plant Sci., 2: 144–151.

Cunningham, S. D.; Anderson, T. A.; Schwab, A.P. and Hsu, F. C. (1995). Phytoremediation of soils contaminated with organic pollutants. Advances in Agronomy., 56: 55–114.

Cunningham, S. D.; Stomp, A. M.; Han, K. H.; Wilbert, S. and Gordon, M. P. (1996). Genetic strategies for enhancing phytoremediation. Annals of the New York Academy of Sci., 721: 481–491.

Czuba, M. (1987). Methyl mercury toxicity in plant cultures: Modification of resistance and demethylation by light and /or 2, 4-dichlorophenoxy acetic acid. Ecotoxicol. Environ. Saf., 13: 191–201.

Dargan, K. S., Chillar, R. K. and Bhardwaj, K. K. R. (1975). Green manuring for more paddy in alkali soils. Indian Farming, 25: 13-14.

Delhaize, E.; Kataoka, T.; Hebb, D. M.; White, R. G. and Ryan, P. R. (2003). Genes encoding proteins of the cation diffusion facilitator family that confer manganese tolerance. Plant Physiol., 15: 1131-1142.

Dembitsky, V. (2003). Rhizosphere competitiveness of trichloroethylene-degrading poplar-colonizing recombinant bacteria. Applied and Environ. Microb., 66: 4673–4678.

Desal, B. K. and Halepyati, A. S. (2007). Growth of *Sesbania grandiflora* and *Sesbania sesban* as influenced by plant densities and phosphorus levels with and without vesicular arbuscular mycorrhiza. Karnataka J. Agric. Sci., 20: 702-705.

Dhankher, O. P.; Li, Y.; Rosen, B. P.; Shi, J.; Salt, D.; Senecoff, J. F; Sashti, N. A. and Meagher, R. B. (2002). Engineering tolerance and hyperaccumulation of arsenic in plants by combining arsenate reductase and gamma-glutamylcysteine synthetase expression. Nat. Biotech., 20: 1140–1145.

Dickinson, N. M. and Pulford, I. D. (2005). Cadmium phytoextraction using short-rotation coppice. Salix: the evidence trail. Environ. Intern., 31: 609–613.

Doty, S. L, James; C. A.; Moore, A. L.; Vajzovic, A.; Singleton, G. L.; Ma, C.; Khan, Z.; Xin, G.; Kang, J. W. and Park, J. Y. (2007).

Enhanced phytoremediation of volatile environmental pollutants with transgenic trees. Proc. Nature Acad. Sci., 104: 16816–16821.

Doty, S. L; Christina, M. Y.; Ingridlundin, J. and Joe, D. (1996). Mutational analysis of the input domain of the virA protein of *Agrobacterium tumefaciens*. J. of Bacteriology, 8: 961–970

Doty, S. L. (2008). Enhancing phytoremediation through the use of transgenics and endophytes. New Phytol., 179: 318-333.

Doty, S. L.; Shang, Q. T.; Wilson, A. M.; Moore, A. L.; Newman, L. A.; Strand, S.E. and Gordon, M. (2003). Metabolism of halogenated by the tropical leguminous tree, *Leucaena leucocephala*. Water Research, 37: 441–449.

Doty, S. L.; Shang, Q. T.; Wilson, A. M.; Tangen, J.; Westergreen, A.; Newman, L. A.; Strand, S. E. and Gordon, M. P. (2000). Enhanced metabolism of halogenated hydrocarbons in transgenic plants containing mammalian P450 2E1. Proc. Nature Acad. Sci., 97: 6287–6291.

Doty, S. L; Dosher, M. R.; Singleton, G. L.; Moore, A, L.; van Aken, B.; Stettler, R. F.; Strand, S. E.; Gordon, M. P. (2005). Identification of an endophytic Rhizobium in stems of *Populus*. Symbiosis, 39: 27–36.

Durst, F. (1991). Biochemistry and physiology of plant cytochrome P-450. In Ruckpaul K (ed) Frontiers in Biotransformation, 4: 191–232.

Dushenkov, S. (2003). Trends in phytoremediation of radionuclides. Plant and Soil, 249: 167–175.

Dushenkov, S.; A. Mikheev, A.; Prokhnevsky, M.; Ruchko, and Sorochinsky, B. (1999). Phytoremediation of radiocesium-contaminated soil in the vicinity of chernobyl, Ukraine. Environ. Sci. and Techn., 33: 469-475.

Dushenkov, S.; Vasudev, Y.; Kapulnik, D.; Gleba, D.; Fleisher, K.; Ting, R. and Ensley, B. (1997). Removal of Uranium from water using terrestrial plants. Environ. Sci. and Techn., 12: 3468-3474.

Eapen, S. and D'Souza, S. F. (2005). Prospects of genetic engineering of plants for phytoremediation of toxic metals. Biotech. Advances, 23: 97–114.

Ebbs, S. D.; Lasat, M. M.; Brady, D. J.; Cornish, J.; Gordon, R. and Kochian, L.V. (1997). Phytoextraction of cadmium and zinc from a contaminated soil. J. of Environ. Quality, 26: 1424–1430.

Edwards, R.; Dixon, D. P. and Walbot, V. (2000). Plant glutathione S-transferases: Enzymes with multiple functions in sickness and in health. Trends Plant Sci., 5: 193–198.

Eide, D.; Broderius, M.; Fett, J. and Guerinot, M. L. (1996). A novel iron-regulated metal transporter from plants identified by functional expression in yeast. Proc. Nature Acad. Sci., 93: 5624-5628.

Entry, J. A.; Watrud, L. S. and Reeves, M. (1999). Accumulation of ^{137}Cs and ^{90}Sr from contaminated soil by three grass species inoculated with mycorrhizal fungi. Environ. Pollution, 104: 449-457.

Entry, J. A.; Watrud, R. S.; Manasse, L. S. and Vance, N. C. (1997). Phytoremediation and reclamation of soils contaminated with radionuclides. American Chem. Soci., 22: 299-306.

Evans, D. E.; Briars, S. A. and Williams, L. E. (1991). Active calcium transport by plant cell membranes. J. Exp. Bot., 42: 285-303.

Evans, D. O. (2001). *Sesbania grandiflora* : NFT for beauty, food, fodder and soil improvement. Agro. Forestry Species and Technologies, 8: 155-156.

Fonné-Pfister, R.; Gaudin, J.; Kreuz, K.; Ransteiner, K. and Eber, E. (1990). Hydroxylation of primisulfuron by an inducible cytochrome P-450-dependent monooxygenase system from maize. Pesticide Biochemistry and Physiology, 37: 165–175.

Fox, T. C. and Guerinot, M. L. (1998). Molecular biology of cation transport in plants. Ann. Rev. Plant Physiol., 49: 669-696.

Goel, A.; Kumar, G.; Payne, G. F., and Dube, S. K. (1997). Plant cell biodegradation of a xenobiotic nitrate ester, nitroglycerin. Nat. Biotech., 15: 174–177.

Gordon, M. P.; Choe, N.; Duffy, J.; Ekuan, G.; Heilman, P.; Muiznieks. I.; Ruszaj, M.; Shurtleff, B. B.; Strand, S. E. and Wilmoth, J. (1998). Phytoremediation of trichloroethylene with hybrid poplars. Environ. Health Perspectives, 106: 1001–1004.

Grangeiro, T. B.; Jorge, D. M., Bezerra,W. M.; Vasconcelos, A. T. R. and Simpson, A. J. G. (2004). Transport genes of Chromobacterium violaceum: An overview. Genetics and Molecular Research, 3: 117–133.

Grotz, N.; Fox, T.; Connolly, E.; Park, W.; Guerinot, M. L. and Eide, D. (1998). Identification of a family of zinc transporter genes from Arabidopsis that respond to zinc deficiency. Proc. Nature Acad. Sci. 95: 7220–7224.

Guengerich, F. P. (1991). Role of human cytochrome P-450 IIE1 in the oxidation of many low molecular weight cancer suspects. Chem. Res. Toxicol., 4: 168–179.

Hammer, D.; Kayser, A. and Keller, C. (2003). Phytoextraction of Cd and Zn with Salix viminalis in field trials. Soil Use Manag., 19: 187–192.

Han, K. H.; Meilan, R.; Ma, C. and Strauss, S. H. (2000). An *Agrobacterium tumefaciens* transformation protocol effective on a

variety of cottonwood hybrids (genus *Populus*). Plant Cell Reports, 19: 315–320.

Hannink, N.; Rosser, S. J. and Bruce, N. C. (2002). Phytoremediation of explosives. Critical Reviews in Plant Sci., 21: 511–538.

Hansikova, H.; Frei, E.; Anzenbacher, P. and Stiborova, M. (1994). Isolation of plant cytochrome P450 and NADPH: Cytochrome P-450 reductase from tulip bulbs (*Tulipa fosteriana*). General Physiology and Biophysics, 13: 149–169

Harms, H. (1996). Bioaccumulation and metabolic fate of sewage sludge derived organic xenobiotics in plants. Sci. of the Total Environ., 185: 83–92.

Health Canada, (2004). Trichloroethylene in drinking water, document for public comment. prepared by the Federal-Provincial-Territorial committee on drinking water. 4:6-16.

Heering, J. H. and Gutteridge, R. C. (1992). *Sesbania grandiflora* (L.). Plant of resoutces of South- East Asia, 4: 196-198.

Hiei, Y., Komari, T. and Kubo T. (1994). Efficient transformation of rice. P. Mol. biology, 2: 271-282.

Hiei, Y., Komari, T. and Kubo T. (2004). Transformation of rice mediated by *Agrobacterium tumefaciens*. Plant Molecular biology, 35: 205-218.

Hirose, S.; Kawahigashi, H.; Ozawa, K.; Shiota, N.; Inui, H.; Ohkawa, H. and Ohkawa, Y. (2005). Transgenic rice containing human CYP2B6 detoxifies various classes of herbicides. J. of Agri. and Food Chem., 53: 3461–3467.

Holton, T. A. and Cornish, E. C. (1995). Genetics and biochemistry of anthocyanin biosynthesis. Plant Cell, 7: 1071–1083.

Hughes, J. B.; Shanks, J.; Vanderford, M.; Lauritzen, J. and Bhadra, R. (1997). Transformation of TNT by aquatic plants and plant tissue cultures. Environ. Sci. and Techn., 31: 266–271.

Huw, D. J.; Angela, D. and Huixia, W. (2005). Review of methodologies and a protocol for the *Agrobacterium*-mediated transformation of wheat. Cereal Res Commun., 31: 9-16.

Hwei-Ming, P. and coon, J. (1998). Regulation of Rabbit cytochrome P450 2E1 expression in HepG2 cells by insulin and thyroid hormone. Molecular Pharmacology, 54: 740–747.

IAEA, (1999). Radionuclide analysis. Technical report series No. 295. Vienna.

Inui, H.; Shiota, N.; Motoi, Y.; Inoue, T.; Ohkawa, Y. and Ohkawa, H. (2001). Metabolism of herbicides and other chemicals in human cytochrome P450 species and in transgenic potato plants coexpressing human CYPA1, CYP2B6, and CYP2C19. J. of Pesticide Sci., 26: 28–40.

Jalal, P.; Pourahmad ; Monireh, G.; Hossein, A. and Ruhollah, G. (2006). A search for cellular and molecular mechanisms involved in depleted uranium (DU) toxicity. Intern. Symposium on Toxicity Assessment, 4: 349-352.

Jefferson, (1987). Assaying chimeric genes in plants: the *GUS* gene fusion system, Plant Mol. Biol. Rep., 4: 387–405.

Jenings, J. C.; Coolbaugh, R. C.; Nakata, D. A. and West, C. A. (1993). Characterization and solubilization of kaurenoic acid hydroxylase from *Gibberella fujikuoro*. Plant Physiology, 101: 925–930.

Jianwei, H.; Jianjun, W.; Berti, and Cunningham, U. (1997). Phytoremediation of lead-contaminated soils: role of synthetic chelates in lead phytoextraction. Environ. Sci. Techno., 31: 800-805.

Kassel, A. G.; Ghoshal, D. and Goyal, A. (2002). Phytoremediation of trichloroethylene using hybrid poplar. Physiology and Molecular Biology of Plants, 8: 1-8.

Kemal, T.; ERCAN, A. and Kenan,T.(1999) *Agrobacterium tumefaciens* Mediated Transformation of Sesame (*Sesamum indicum L.*). Tr. J. of Botany, 23:291-295

Khan, (2003). Clipping inoculation method for evaluating quantitative resistance of rice varieties to bacterial leaf blight. Kinki-Chugoku Agric. Res., 5: 89-95.

Knops, M.; Schphan, I. and Schmidt, B. (1995). Biotransformation of 4-nitrophenol by a fermenter grown cell suspension culture of Soybean (*Glycine max*): Isolation and identification of conjugates. Plant Sci., 109: 215–224.

Kochs, G. and Grisebach, H. (1986). Enzymatic synthesis of isoflavonoids. European J. of Biochemistry, 155: 311–318.

Kozhevnikova, A. D.; I. V. Seregin, E. I.; Bystrova, and V. B. Ivanov, P. (2006). Effects of heavy metals and strontium on division of root cap cells and meristem structural organization. Russian J. of Plant Physiology, 8: 257-266.

Kramer, U. and Chardonnens, A. N. (2001). The use of transgenic plants in the bioremediation of soils contaminated with trace elements. Applied Microb. Biotech., 55: 661–672.

Kunkel, B. N. (1996). A useful weed put to work: genetic analysis of disease resistance. Plant Cell, 11: 1159–1161.

Kutchan, T. M. (1995). Alkaloid biosynthesis the basis for metabolic engineering of medicinal plants. Plant Cell, 7: 1059–1061.

Kuzovkina, Y. A. and Quigley, M. F. (2005). Willows beyond wetlands: uses of Salix L. species for environmental projects. Water, Air, and Soil Pollution, 162: 183–204.

Laibach, F. (1943). Cell divisions in cotyledons after germination: localization, time course and utilization for a mutagenesis assay. Planta., 199: 307-313.

Laurie, S.; Feeney, K. A.; Maathuis, F. J. M.; Heard, P. J.; Brown, S. J. and Leigh, R. A. (2002). A role for HKT1 in sodium uptake by wheat roots. Plant J., 32: 139-149.

Lee, S., Moon; J. S., Domier, L. L. and Korban, S. S. (2003). Molecular characterization of phytochelatin synthase expression in transgenic *Arabidopsis*. Plant Physiol. Biochem, 40: 727-733.

Legge, R. L.; Thompson, E.; Baker, J. E. and Lieberman, M. (1982). The effect of calcium on the fluidity and phase properties of microsomal membranes isolated from post climacteric Golden delicious apples. Plant Cell Physiol., 23: 161-169.

Li, L.; Zhou, Y.; Cheng, X.; Sun, J.; Marita, J. M.; Ralph, J. and Chiang, V. (2003). Combinatorial modification of multiple lignin traits in trees through multigene cotransformation. Proc. Nature Acad. Sci., 100: 4939–4944.

Liang, Z.Y.; Pilon-Smits, E. A.; Jouanin, L. and Terry, N. (1999). Overexpression of glutathione synthetase in Indian mustard enhances cadmium accumulation and tolerance. Plant Physiology, 119: 73–80.

Liao, M.T.; Hedley, M. J.; Woolley, D. J.; Brooks, R. R. and Nichols, M. A. (2000). Copper uptake and translocation in chicory (*Cichorium intybus* L. cv *Grasslands Puna*) and tomato (*Lycopersicon esculentum* Mill. Cv Rondy) plants grown in NFT system: II. The role of nicotianamine and histidine in xylem sap copper transport. Plant Soil, 223: 243-252.

Lin, J.; Assad-Garcia, N. and Kno, J. (1995). Plant hormone effect of antibiotics on the transformation efficiency of plant tissues by *A. tumefaciens* cells. Plant Sci., 109: 171-177.

Lodewyckx, C.; Taghavi, S.; Mergeay, M.; Vangronsveld, J.; Clijsters, H. and van der, L. D. (2001). The effect of recombinant heavy metal resistant endophytic bacteria in heavy metal uptake by their host plant. International J. of Phytoremediation, 3: 173–187.

Lu, Y. P.; Li, Z. S.; Drozdowicz, Y. M.; Hortensteiner, S.; Martinoia, E. and Rea, P. A. (1998). AtMRP2, an Arabidopsis ATP binding cassette transporter able to transport glutathiones-conjugates and chlorophyll catabolites: Functional comparisons with AtMRP1. Plant Cell, 10: 23-18

Ma, X. and J. G. Burken, (2002). VOCs Fate and partitioning in vegetation: Use of tree cores in groundwater analysis. Environ. Sci. Technol., 36: 4663-4668.

Ma, X. and J. G. Burken, (2003). TCE diffusion to the atmosphere in phytoremediation applications. Environ. Sci. Technol., 37: 2534-2539.

Mackay, D.; Shiu, H. and Ma, K. (1993). Illustrated handbook of physical-chemical properties and environmental fate for organic chemicals, VolII: CRC. P**ress.**

Malik, K. A. and Haider, K. (1977). Decomposition of carbon-14-labelled plant material in saline-sodic soils. In: Soil Organic Matter Studies. International Atomic Energy Agency, Vienna., 3: 215-225.

Maniatis, T.; Fritch, E. F. and Sambrook, J. (1982). Molecular cloning. A laboratory manual. Cold Spring Harbour. NewYourk.

Marrs, K. A. (1996). The functions and regulation of glutathione S-transferases in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol., 47: 127–158.

McIntyre, H. C. (2003). The effect of photoperiod and temperature on growth and frost resistance of winter rye root systems. Plant Physiol., 79: 519-525.

Meagher, R. B. and Heaton, A. C. (2005). Strategies for the engineered phytoremediation of toxic element pollution: mercury and arsenic. J. of Industrial Microb. and Biotech., 32: 502–513.

Meyerowitz, E. M. (1989). *Arabidopsis*, a useful weed. Cell, 56: 263-269.

Miller, J. H. **(**1998). Experiments in Molecular Genetics, p. 21. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Morant, M.; Bak, S.; Moller, B. L. and Werck-Reichhart, D. (2003). Plant cytochromes P450: tools for pharmacology, plant protection and phytoremediation. Current Opinnion in Biotech., 2: 151-162.

Moser, B. W.; Pipas M. J.; Witmer, G. W. and Engeman, R. M. (2002). Small mammal use of hybrid poplar plantations relative to stand age. Northwest Sci., 76: 158–165.

Mougin, C.; Cabanne, F.; Canivenc, M. C. and Scalla, R. (1990). Hydroxylation and Ndemethylation of chlortoluron by wheat microsomal enzymes. Plant Sci. Letters, 66: 195–203.

Munns, R.; Greenway, H. and Kirst, G. O. (1983). Halotolerant eukaryotes. Encyclopedia of Plant Physiology. 8: 11-15.

Muraoka, K. and Hirata, T. (1988). Hydraulic behavior of chlorinated organic compounds in water. Pak. J. Agric, Res., 22: 485–489.

Murashige, T.and Skoog, (1962). A revised medium for rapid growth and bioassays with tobacco tissues culture, Physiol. Plant, 15, 473-497.

Nandwani, F. and Ramawal K. (1992). Regeneration efficiency and embryogenic callus production of three cultivars of rice. Pak. J. Agric, Res., 34: 66-74.

Natalia, D.; Wang, C. and Zhang, D. (2002). Progress in map based cloning of Xa 22, a new gene for bacterial leaf blight resistance in rice. Paper presented at plant and animal genome conference. 18-22.

Nauerby, B., Billing, K. and Wyndacle, R. (1997). Influence of antibiotic time tin on plant regeneration compared to carbincillin and cefotaxine in concentration b5 suitable for elimination of *Agrobacterium tumefaciens*. Plant Sci. Limerick, 123: 169-177.

Nedelkoska, T. V. and Doran, P. (2000). Hyperaccumulation of cadmium by hairy roots of *Thlaspi caerulescens*. Biotech. and Bioengineering, 67: 607–615.

Negri, C. M., and Hinchman, R. R. (2000). The use of plants for the treatment of radionuclides. Wiley-Interscience Publication, 3: 77-83.

Nelson, D. E., Koukoumanos, M. and Bohnert, H. J. (1983). Myo-inositol dependent sodium uptake in ice plant. Plant Physiol., 119: 165-172.

Newman, L. A. and Reynolds, C. M. (2005). Bacteria and phytoremediation: new uses for endophytic bacteria in plants. Trends in Biotech., 23: 6–8.

Newman, L.; Strand, S.; Choe, N.; Duffy, J.; Ekuan, G.; Ruszaj, M.; Shurtleff, B.; Wilmoth, J.; Heilman, P. and Gordon, M.P. (1997). Uptake and biotransformation of trichloroethylene by hybrid poplars. Environ. Sci. and Techn., 31: 1062–1067.

Newman, L.; Wang. X.; Muiznieks, I.; Ekuan, G.; Ruszaj, M.; Cortellucci, R.; Domroes, D.; Karscig, G.; Newman, T. and Crampton, R. S. (1999). Remediation of trichloroethylene in an artificial aquifer with trees: a controlled field study. Environ. Sci. and Techn., 33: 2257–2265.

Njoroge, G. and Bussmann, N. (2008). Diversity and utilization of antimalarial ethnophytotherapeutic remedies among the Kikuyus (Central Kenya). J. Ethnobiol. Ethnomed., 2: 8–14.

Noctor, G.; Strohm, M.; Jouanin, L.; Kunert, K. J.; Foyer, C. H. and Rennenberg, H. (1996). Synthesis of glutathione in leaves of transgenic poplar overexpressing [gamma]-glutamylcysteine synthetase. P. Phys., 112: 1071–1078.

Onim, J. F. and Dzowela, B. H. (2006). The distribution of *Sesbania spp* in the Pansea region, 8: 562–573.

Onim, J. F. M. (1986). Nitrogen fixation in legumes. Proceedings of a conference held at ILCA, Addis Ababa, Ethiopia, 4: 35-40.

Onim, J. F. M.; Semenye, P. P. and Fitzhugh, H. A. (1985). Small ruminants in African agriculture. Proceedings of a conference held at ILCA, Addis Ababa, Ethiopia, 4: 44-49.

Osta, R. J. (2004). Environmentally acceptable endpoints in soil. American Academy of Environ. Engineers, 106: 66-75.

Osta, R. J. (2007). Mode of action of liver tumor induction by trichloroethylene and its metabolites, trichloroacetate and dichloroacetate. Environ. Health Perspect., 108: 241–259.

Parameswaran, A.; Leitenmaier, B.; Yang, M.; Kroneck, P. M.; Welte, W.; Lutz, G.; Papoyan, A.; Kochian, L. V. and Kupper, H. (2007). A native Zn/Cd pumping P (1B) ATPase from natural overexpression in a hyperaccumulator plant. Bioch. and Biophysical Research Communications, 363: 51–56.

Paul, C. and Campanella, B. (2000). Novel gene expression profiles define the metabolic and physiological processes characteristic of wood and its extractive formation in a hardwood tree species, *Robinia pseudoacacia*. Plant Mol. Biol., 52: 935–956.

Pence, N. S.; Larsen, P. B.; Ebbs, S. D.; Letham, D. L.; Lasat, M. M.; Garvin, D.F.; Eide, D. and Kochian, L. V. (2000). The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. Proc. Nature Acad. Sci., 97: 4956–4960.

Pflugmacher, S. and Sandermann, H. (1992). Cytochrome P450 monooxygenases for fatty acids and xenobiotics in marine microalgae. Plant Physiol., 117: 123–128.

Pierrel, L.; Guillet, K. and Busvine, J. R. (1994). The significance of DDT-irritability testing on mosquitoes. Bulletin of the World Health Organization, 31: 645-656

Pilon, M.; Owen, J. D.; Garifullina, G. F.; Kurihara, T.; Mihara, H.; Esaki, N. and Pilon-Smits. E. H. (2003). Enhanced selenium tolerance and accumulation in transgenic *Arabidopsis* expressing a mouse selenocysteine lyase. Plant Physiology, 131: 1250–1257.

Pilon-Smits, E. H. and Pilon, M. (2002). Phytoremediation of metals using transgenic plants. Critical Reviews in Plant Sci., 21: 439–456.

Polisetty, Y.; Kuhn, A. and Wittig, R. (1997). Enhanced Selenium tolerance and accumulation in transgenic Arabidopsis expressing a mouse selenocysteine lyase. Plant Physiology, 131: 1250–1257.

Pushnik, J. C and Miller, G. W. (1989). Iron regulation of chloroplast photosynthetic function: Mediation of PSI development. J. Plant Nutr., 12: 407-421.

Priya, P. and Shivendra V. (2009). Genetic transformation and regeneration of *Sesbania drummondii* using cotyledonary nodes. Plant Cell Rep., 28:31–40.

Rashied, H.; Bukhari, S. and Qureshi, A. (1996). Transgenic plant production mediated by Agrobacterium in indica rice. Plant Cell Rep., 15: 727-730.

Reid, R. J. and Smith, F. A. (2000). The limits of sodium/calcium interactions in plant growth. J. Plant Physiol., 27: 709-715.

Reinhold-Hurek, B. and Hurek, T. (1998). Life in grasses: diazotrophic endophytes. Trends in Microb., 6: 139–144.

Rivera, R; Medina V. F.; Larson S. L. and McCutcheon, S. C. (1998). Phytotreatment of TNT-contaminated groundwater. J. of Soil Contamination, 7: 511–529.

Robineau, T.; Batard, Y.; Nedelkina, S.; Cabello-Hurtado, F.; LeRet, M.; Sorokine, O.; Didierjean, L. and Werck-Reichhart, D. (1998). The chemically inducible plant cytochrome P450 CYP76B1 actively metabolizes phenylureas and other xenobiotics. Plant Physiology, 118: 1049–1056.

Robinson, B. H.; Mills, T. M.; Petit, D.; Fung, L. E.; Green, S. R. and Clothier, B. E. (2000). Natural and induced cadmium accumulation in poplar and willow: implications for phytoremediation. Plant and Soil, 227: 301–306.

Romantschuk, M.; Sarand, I.; Petanen, T.; Peltola, R.; Jonsson-Vihanne, M.; Koivula, T.; Yrjala, K.; Haahtela, K. (2000). Means to improve the effect situ bioremediation of contaminated soil: an overview of novel approaches. Environ. Pollution, 107: 179–185.

Rosenblatt, D. H. (1980). Toxicology of explosives and propellants. In: Kaye SM, ed. Encyclopedia of explosives and related items. US Army Armament Research Development Committee, 3: 332–345.

Rosser, S. J.; French, C. E. and Bruce, N. C. (2001). Engineering plants for the phytoremediation of explosives. *In Vitro* Cellular and Developmental Biology Plant, 37: 330–333.

Rsoc, (1999). Resolving the DDT dilemma: Protecting biodiversity and human health. World Wildlife Fund Canada, 10: 295–299.

Rugh, C. L. (2004). Genetically engineered phytoremediation: one man's trash is another man's transgene. Trends in Biotech., 22: 496–498.

Rupasinghe, S.; Duan, H. and Schuler, M. A. (2007). Molecular definitions of fatty acid hydroxylases in *Arabidopsis thaliana*. Proteins, 68: 279–93.

Safferman, S. I.; Lamar, R. T.; Vonderhaar, S.; Neogy, R.; Haught, R. C.; and Krishnan, L. (1995). Treatability study using phanerochaete sordid for the bioremediation of DDT contaminated soil. Toxicol. and Environ. Chem., 50: 237-251.

Salishary, G., and Ross, R.W. (1982). Isolation, chromosomal localization, and differential expression of mitochondrial manganese superoxide dismutase and chloroplastic copper/zinc superoxide dismutase genes in wheat. Plant Physiol., 120: 513-520.

Salt, D. E. and Kramer, U. (1998). Mechanisms of metal hyperaccumulation in plants. In: Phytoremediation of toxic metals: using plants to clean-up. Environment, 12: 231–246.

Salt, D. E.; Smith, R. D. and Raskin, I. (1997). Phytoremediation. Annu. Rev. Plant Physiol. Plant Mol. Biol., 49: 643-668.

Sandermann, H. (1992). Plant metabolism of xenobiotics. Trend in Biotechnol., 17: 82–84.

Sandermann, H. (1994). Higher plant metabolism of xenobiotics: the "green liver" concept. Pharmacogenetics, 4: 225–241.

Sandmann, G. and Boger, P. (1983). The enzymatological function of heavy metals and their role in electron transfer processes of plants. In: Inorganic Plant Nutrition, Encycl, Plant Physiol., 15: 563-596.

SAS (2001). SAS/STAT Users Guide for Personal Computers. Release 6.12, SAS Inst. Inc. Nc. USA.

Schaaf, G.; Ludewig, U.; Erenoglu, B. E.; Mori, S.; Kitahara, T. and von Wiren, N. (2004). ZmYS1 functions as a proton-coupled symporter for phytosiderophore- and nicotianamine-chelated metals. J. Biol. Chem., 279: 9091-9096.

Schaap, A P., Akhavan-Tafti, H , and Romano, L. J (1989) Chemiluminescent substrates for alkaline phosphatase: application to ultrasensitive enzyme-linked immunoassays and DNA probes Clw. Chem 35, 1863,1864.

Schachtman, D. P.; Kumar, R.; Schroeder, J. I. and Marsch, E. L. (1997). Molecular and functional characterization of a novel low-affinity cation transporter (LCT1) in higher plants. Proc. Nat. Acad. Sci., 94: 11079-11084.

Schachtman, D. P.; Tyerman, S. D. and Terry, B. R. (1991). The K^+/Na^+ selectivity of a cation channel in the plasma membrane of root cells does not differ in salt-tolerant and salt-sensitive wheat species. Plant Physic., 97: 598-605.

Schnoor, J. L.; Licht, L. A.; McCutcheon, S.; Wolfe, N. L. and Carreira, L. H. (1995). Phytoremediation of contaminated soils and sediments. Environ. Sci. and Techn., 29: 318–323

Schoenmuth, B. W. and Pestemer, W. (2004). Dendroremediation of trinitrotoluene (TNT). Part 2: fate of radio-labelled TNT in trees. Environ. Sci. and Pollution Research, 11: 331–339.

Schuler, M. A. (1996). Plant cytochrome P450 monooxygenases. Critical Reviews in Plant Sci., 15: 235–284.

Schwab, A. P.; Al-Assi, A. A. and Banks, M. K. (1998). Adsorption of naphthalene plant roots. J. of Environ. Quality, 27: 220–224.

Schwille, F. (1988). Dense chlorinated solvents in porous and fractured media. Lewis Publishers, New York, 5: 157- 161.

Scott, N. C. (1999). Toxicology of maternally ingested trichloroethylene (TCE) on embryonal and fetal development in mice and of TCE metabolites on in vitro fertilization. Fundam. Appl. Toxicol., 2: 268–274.

Selosse, M. A.; Baudoin, E. and Vandenkoornhuyse, P. (2004). Symbiotic microorganisms, a key for ecological success and protection of plants. Comptes Rendus Biologies., 327: 639–648.

Shang, Q. T. and Gordon, M. P. (2002). Transformation of [14C] trichloroethylene by poplar suspension cells. Chemosphere, 47: 957–962.

Shang, Q. T; Doty, S. L; Wilson, A. M.; Howald, W. N. and Gordon, M.P. (2001). Trichloroethylene oxidative metabolism in plants: the trichloroethanol pathway. Phytochemistry, 58: 1055–1065.

Shim, H.; Chauhan, S.; Ryoo, D.; Bowers, K.; Thomas, S. M.; Canada, K. A.; Burken J. G. and Wood, T. K. (2000). Rhizosphere competitiveness of trichloroethylene-degrading, poplar-colonizing recombinant bacteria. Applied and Environ. Microb., 66: 4673–4678.

Siciliano, S. D.; Fortin, N.; Mihoc, A.; Wisse, G.; Labelle, S.; Beaumier, D.; Ouellette, D.; Roy, R.; Whyte, L. G. and Banks, M. K. (2001). Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. Applied and Environ. Microb., 6: 2469–2475.

Song, W. Y.; Sohn, E. J.; Martinoia, E.; Lee, Y. J.; Yang, Y. Y.; Jasinski, M.; Forestier, C.; Hwang, I. and Lee, Y. (2003). Engineering tolerance

and accumulation of lead and cadmium in transgenic plants. Nat. Biotech., 21: 914–919.

Srivastava, G. P.; Bhoi, R. L. and Khan, M. R. (1973). Reclamation of saline soil by a mixture of dhaincha and superphosphate. Allahabad Farmer, 47: 43-47.

Stanton, B.; Eaton, J.; Johnson, J.; Rice, D.; Schuette, B. and Moser, B. (2002). Hybrid poplar in the Pacific Northwest. J. of Forestry, 100: 28–33.

Steven, B. J; Veech R. L, and Saeng (2008). Cytochrome P450IIE1 is elevated in lymphocytes from poorly controlled insulin-dependent diabetics. J. Clin. Endocrinol. Metab., 71: 1036–1040.

Steven, J. C., and Andrew F. B. (1998). Floral dip simplified Methods for *Agrobacterium transformation*. The plant J., 16: 735- 743.

Stode, S. E.; Ong, C. N.; Tsakok, M. F. and Ho, A. (2006). Semen parameters in workers exposed to trichloroethylene. Reprod. Toxicol., 10: 295–299.

Sussman, M. R.; Amasino, R.; Young, J.C.; Krysan, P. J. and Austin-Phillips, S. (2000). DNA methylation profiling identifies CG methylation clusters in *Arabidopsis* genes. Curr. Biol., 15:154–159.

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 improves phytoremediation of toluene. Applied and Environ. Microb., 71: 8500–8505.

Tester, M. and Davenport, D. (2003). Na^+ tolerance and Na^+ transport in higher plants. Ann. Bot., 91: 503-527.

Thakur, A.; Sarita, S. and Srivastava, D.(2005). Plant regeneration and genetic transformation studies in petiole tissue of Himalayan poplar (*Populus ciliata* Wall.). Current Science, 89: 664-668.

Thompson, P. L.; Ramer, L. A. and Schnoor, J. L. (1998). Uptake and transformation of TNT by hybrid poplar trees. Environ. Sci. and Techn., 32: 975–980.

Tisdale, S. L.; Nelson, J. D.; Beaton, L. and Havlin, J. L. (1997). Soil Fertility and Fertilizers. 5th. Ed Macmillan Publ. Co. New York, NY, USA.

Toki, S. (1997). Rapid and efficient *Agrobacterium*–mediated transformation in rice. Plant Mol. Gen. Gent., 220: 389-392.

Travis, E. R.; Hannink, N. K.; Van der Gast, C. J.; Thompson, I. P.; Rosser, S. J. and Bruce, N. C. (2007). Impact of transgenic tobacco on trinitrotoluene (TNT) contaminated soil community. Environ. Sci.and Techn., 41: 5854–5861.

Trenck, K. T. and Sandermann, H. J. (1979). Metabolism of benzo[a]pyrene in cell suspension cultures of parsley (*Petroselimom hortense* Hoffm.) and soybean (*Glycine max* L.). Planta, 4: 245–251.

Tsao, D. T. (2003). Overview of phytotechnologies. Advances in Biochemical Engineering and Biotech., 78: 1–50.

USEPA (United States Environmental Protection Agency) (2001). Trichloroethylene health risk assessment: Synthesis and characterization. Office of Research and Development, 7: 546-551.

USEPA, (United States Environmental Protection Agency) (1998). A citizen's guide to phytoremediation. Office of Solid Waste and Emergency Response, EPA, 3: 542-555.

Veluthamble, K.; Krishnan, M. and gould, j. H. (1989). Opines stimulate induction of vir genes of the *Agrobacterium tumefaciens* Ti plasmid. J. of Bacteriology, 70: 3696-3703.

Vido, K. A. (2001). Proteome analysis of the cadmium response in *Saccharomyces cerevisiae*. J. of Biological Chemistry, 276: 8469–8474.

Wang, G. D.; Li, Q. J.; Luo, B. and Chen, X. (2004). Explanta phytoremediation of trichlorophenol and phenolic allelochemicals via an engineered secretory laccase. Nat. Biotech., 22: 893–897.

Wang, X.; Gordon, M. P. and Strand, S. E. (2002). Mechanism of aerobic transformation of carbon tetrachloride by poplar cells. Biodegradation, 13: 297–305.

Whalen, M., Innes, Bent, A. and staskawicz, B. (1991). Identification of Pseudmonas Syringae pathogens of *Arabidopsis thalania* and a bacterial gene determining avirulence on both Arabidopsis and Soybean. Plant Cell., 3: 49-59.

Whetten, R. and Sederoff, R. (1995). Lignin biosynthesis. Plant Cell, 7: 1001–1013.

WHO, (1999). Guidelines for Drinking Water Quality. Vol. 2. Recommendations. World Health Organization, Geneva.

Widdowson, M. A.; Shearer, S.; Andersen, R. G. and Novak, J. T. (2005). Remediation of polycyclic aromatic hydrocarbon compounds in groundwater using poplar trees. Environ. Sci. and Techn., 39: 1598–1605.

Wilson, Z. A. (2000). Arabidopsis a practical approach. Oxford University Press, Inc., 22: 47-52.

Wittig, R., Ballach, H. J. and Kuhn, A. (2003). Exposure of the roots of *Populus nigra* L. cv. Loenen to PAHs and its effect on growth and water balance. Environ. Sci. and Pollution Research, 10: 235–244.

Wu, D., and Cenderbaim, A. I. (2001). Presence of functionally active cytochrome P-450IIE1 in the plasma membrane of rat hepatocytes. Hepatology, 15: 515-524.

Yousir, (2008) *In vivo* and *in vitro* studies on heavy metal tolerance in *Sesbania grandiflora* L. M.Sc. thesis, Univ. of AL-Nahrain, Baghdad, Iraq.

Zhang, W.; Cai, Y.; Tu, C. and Ma, L. Q. (2002). Arsenic speciation and distribution in an arsenic hyperaccumulating plant. Sci. of the Total Environment, 300: 167–177.

Zhu, Y. L.; Pilon-Smits, E. A; Tarun, A. S.; Weber, S. U.; Jouanin, L. and Terry, N. (2002). Cadmium tolerance and accumulation in Indian mustard is enhanced by overexpressing gamma-glutamylcysteine synthetase. Plant Physiology, 121: 1169–1178.

Zhuang, X.; Chen, J.; Shim, H. and Bai, Z. (2007). New advances in plant growth-promoting rhizobacteria for bioremediation. Environment International, 33: 406–413.

Zumdahl, S. S. (1989). Atoms, Molecules, and Ions. Chapter 2 of Chemistry. 2nd ed. Lexington: D. C. Health and Company.

الخلاصة

هدفت الدراسة الحالية إلى زيادة قابلية نباتي السيسبان *Sesbania grandiflora* و الاربيدوبسيس *Arabidopsis thaliana* المعالجة النباتية للملوثات من خلال نقل المورث P4502E1 إلى النباتين بوساطة البكتيريا *Agrobacterium tumefaciens* .

السايتوكروم P450 2E1 هو انزيم سيطرة وتحدد عمليات ايض العديد من الملوثات البيئية، وتتضمن ثنائي كلورا ثنائي فنيل ثلاثي كلوراثلين و ثلاثي كلوراثلين.

بينت النتائج بان النسبة المئوية من التحوير لنبات السيسبان ازدادت مع زيادة تركيز المعلق البكتيري مما زاد فرصة الاصابة للجزء النباتي.

تراوحت نسب الاستخلاص النباتي لمبيدDDT من ٥.٧ الى ٩.٣ ميكروغرام لكل مليلتر في نبات الاربيدوبسيس ونبات السيسبان المعبرة وراثيا على التوالي بعد اثنى عشر يوم من بدء التجربة.

ارتبط ايض الملوث الكلوري العضوي ثلاثي كلوروايثلين (TCE) بالنوع النباتي لنباتي السيسبان والاربيدوبسس. ثلاثي كلوراثين ايض بنسب اعلى لثلاثي كلورايثانول(TCEOH) مقارنة" بالكلورل هايدريت (CH) في انسجة نبات الاربيدوبسس بينما حصل العكس في نبات السيسبان. نباتات الاربيدوبسس المعبرة وراثيا كانت اكثر امتصاص للـــ ثلاثي كلوروايثلين مقارنة" مع نباتات السيسبان المعبرة وراثيا.

اختبرت قابلية النباتات المعبرة وراثيا (الاربيدوبسس والسيسبان) لامكانية ازالة العناصر،اظهرت النباتات المعبرة وراثيا بوضوح كفاءة أكثرَ في تراكم عناصر البوتاسيوم ، الكالسيوم ، المنغنيز ، الحديد ، النحاس ، الخارصين ، البروم و الرصاص بنسب ٥٥، ٥٩، ٥٤، ٥٢، ٥١، ٥٤، ٥٨% و٥١% مقارنة" بالنباتاتِ الطبيعية. أبدتْ نباتات الاربيدوبسيس والسيسبان قوة تعبير لجين السايتوكروم P450 قادر على امتصاص السترونتيوم، اليورانيوم والسيزيوم منْ الترَبِ الملوثةِ. كما أظهرت النتائجُ تحمل متزايد لنباتي السيسبان والاربيدوبسيس المعبرة قدر

الإهداء

إلى نور النور ومدبر الأمور ونور السموات والأرض ... فالق الحب والنوى ...الله جل جلاله

إلى أمين الله المأمون .. وخازن علمه المخزون .. وشهيده يوم الدين ورسوله إلى الخلق أجمعين .. حبيب اله العالمين .. محمد المصطفى صلى الله عليه وسلم

إلى جميع الأنبياء والمرسلين والـــ بيت المصطفى معادن التـــقى والسداد ومنبع الهدى والرشاد عليهم السلام أجمعين

إلى ارض الخير والعطاء ... ارض الرافدين ... وطني الغالي ... العراق

إلى التي حملتني وهنا على وهن وغمرتني حنانا وزرعت في نفسي الأمل أمي

إلى من أنبتني غرساَ طيباَ وأنار الدرب أمامي .....أبي

إلى الشموع المضيئة في حياتي ...اخوتي (علاء ورائد ووسام) وأخواتي(يسرى و رنا)

الى رفيق دربي ..... (زوجي سعد)

الى ملائكتي..... (هاشم و سولافه)

إلى رمز الحب والوفاء والإخلاص الصحبة الطيبة ... د. ابراهيم وصفاء و و ايناس واميرة و احمد و رياض و هشام

الى كل من وهب ثمرة جهده في خدمة البشرية وراعى موجودات الأرض كما خلقها الله.

الى من سقوا بدمائهم الطاهرة ارض العراق..... شهداء العراق

اهدي ثمرة جهدي .....رغد

بسْمِ اللَّـهِ الرَّحْمَنِ الرَّحِيمِ

وَإِنْ مِنْ شَيْءٍ إِلاّ عِنْدَنَا خَزَائِنُهُ وَمَا نُنَزِّلُهُ إِلاّ بِقَدَرٍ مَعْلُومٍ

صدق الله العلي العظيم

سورة الحجر اية ٢١

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم

إمكانية بعض النباتات النامية محلياً للتحويل الوراثي بالسايتوكروم P450 وإستعمالها في المعالجة النباتية

اطروحة

مقدمة الى مجلس كلية العلوم – جامعة النهرين

وهي جزء من متطلبات نيل درجة دكتوراه فلسفه في التقانة الاحيائية

من قبل

رغد سلمان محمد

بكالوريوس علوم زراعية - كلية الزراعة ، جامعة بغداد ١٩٩٦

ماجستير علوم زراعية - كلية الزراعة ، جامعة بغداد ٢٠٠٢

تحت اشراف

د. كاظم محمد ابراهيم
استاذ

د. نورية عبد الحسين علي
استاذ

ربيع الاول ١٤٣١هـ

اذار ٢٠١٠ م