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Investigating Heavy Metals Tolerance in *Ruta graveolens In vivo* and *In vitro*

A thesis

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By

Zainab Mahmood Lateef
B.Sc. Biochemical technology, University of Technology (2007)

Supervised by

Dr. Kadhim M. Ibrahim
(Professor)

Dr. Abdulwahid S. Jabir
(Ass. Professor)

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Jamadi al-thani 1436

Supervisors Certification

We, certify that this thesis entitled "**Investigating heavy metals tolerance in *Ruta graveolens in vivo* and *in vitro"*** was prepared by "**Zainab Mahmood Lateef**" under our supervision at the College of Science\ Al-Nahrain University as a partial requirement for the degree of Master of Science in Biotechnology.

Signature:

Supervisor:

Dr. Kadhim M. Ibrahim

Professor

Date: / / 2015

Signature:

Supervisor:

Dr. Abdulwahid S. Jabir

Ass. Professor

Date: / / 2015

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

Signature:

Name: **Dr. Hameed M. Jasim**

Scientific Degree: Professor

Title: Head of Biotechnology Department

Date: / / 2015

Committee Certification

We, the examining committee, certify that we have read this thesis and examined the student in its contents and that, according to our opinion, is accepted as a thesis for the degree of Master of Science in Biotechnology.

Signature:

Name: **Dr. Muayad S. Shawket**

Scientific Degree: Professor

Date:

(Chairman)

Signature:

Name: Dr. **Hussam S. M. Khierallah**

Scientific Degree: Ass. Professor

Date:

(Member)

Signature:

Name: **Dr. Shaymaa H. Al-Rajihi**

Scientific Degree: Lecturer

Date:

(Member)

Signature:

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

Scientific Degree: Professor

Date:

(Member)

Signature:

Name: **Dr. Abdulwahid S. Jabir**

Scientific Degree: Ass. Professor

Date:

(Member)

I hereby certify upon the decision of the examining committee

Signature:

Name: **Dr. Hadi M. A. Abood**

Scientific Degree: Assistant Professor

Title: Dean of College of Science

Date:

Summary

Many experiments were carried out to investigate the ability of *Ruta graveolens* to tolerate heavy metals *in vitro* and to investigate molecular bases of heavy metals tolerance. Callus was induced on stem segment explants cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D) (0.0, 0.75, 1.0 or 1.25 mg/l) and kinetin (kin) (0.0, 0.75, 1.0 or 1.25 mg/l). The combination of 1 mg/l 2,4-D plus 1 mg/l kin and 1.25 mg /l 2,4-D plus 1.00 mg /l kin gave the highest % callus induction reached 100% and 93% respectively. Callus was maintained on MS medium supplemented with 1 mg/l 2,4-D and 0.5 mg/l kin. Callus pieces initiated on stems explants were subcultured into maintenance medium supplemented with manitol at the concentration 300 mg/l to induce drought stress or with saline water at an electrical conductivity (EC) 30 $ds.m^{-1}$ for inducing salt stress. Drought and salt tolerant calli were selected and subjected to heavy metals (Zn, Cd or Fe) at different concentrations (0.0, 0.1, 0.5, 1.0, 2.0 or 3.0 ppm). Results showed that callus fresh weight decreased with increasing metals concentrations. Tolerance index was assessed at different metals concentrations and stress durations. The highest values were recorded under metals concentration 1 ppm after 63 days except in case of salt tolerant calli as it showed its highest tolerance index value after 21 days under Cd stress. Atomic absorption spectrophotometer 5000 was used for estimation metal concentrations inside leaf tissues of plants grown on typical soil and those of stressed and non-stressed callus. Results showed that *R. graveolens* cultures stand for moderately high concentrations of heavy metals (7100 Zn, 440 Fe and 95 μg/g Cd). Heavy metals tolerant calli were selected and shoots were regenerated on 1/2 MS medium supplemented with Benzyl

adenine (BA) (0.0, 2.5 or 3.0 mg/l) and Naphthalene acetic acid NAA (0.0 or 0.5 mg/l). BA at concentration 2.5 mg/l gave the highest % shoot formation after 8 weeks. Shoot elongation and multiplication was achieved on the same regeneration medium enriched with 0.5 mg/l Gibberellic acid (GA3). An examination of regenerated plants for their tolerance to heavy metals was achieved by culturing on MS medium supplemented with heavy metals at concentration 1 ppm. Healthy elongated shoots were cultured on1/2 MS medium supplemented with 0.1 mg/l Indole butyric acid (IBA) to initiate rooting. PCR analysis was performed to investigate molecular basis of heavy metals tolerance in *R. graveolens*. 1-aminocyclopropane-1-carboxylic acid (ACC) synthase gene (ACS2) of sweet orange (*Citrus sinensis*) was selected to design tow primer pairs for PCR assay, so that to detect the presence of the gene in *R. graveolens* plant in control and heavy metal treated plants. The primers were named Pp600 and Pp400. Analysis of PCR product exhibit that control sample produced a PCR product with a molecular size ~ 600 bp using Pp600 primers pair and a PCR product with a molecular size ~ 400 bp using Pp400 primers pair confirming the presence of ACS2 gene. The Effect of abiotic stresses salt, drought and heavy metals on ACS2 gene was also investigated. Results showed that all treatments produced a PCR product with a molecular size ~ 600 bp using specific primers pair Pp600 except in case. Results indicated that samples produced a PCR products with variant molecular sizes using specific primers pair Pp400. Samples under drought and heavy metals (Fe, Zn, and Cd) stresses gave a product with a molecular size ~ 400 bp. *R. graveolens* under salt and Fe stress produced a PCR product ~ 213 bp. Samples under salt and Zn stresses which produced a PCR product with a molecular size ~ 226 bp. Field experiment was carried out to investigate plant tolerance to

heavy metals *in vivo*. It is concluded from the current study, the possibility of increasing Ruta tolerance to heavy metals and then exploitation such tolerant plant for re-vegetation of salt and drought affected lands. The presence of tolerance gene was confirmed.

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List of Abbreviatives

Abbreviation	Full name
2,4-D	2,4-Dichlorophenoxyacetic acid
AtHMA4	*Arabidopsis thaliana* Heavy Metal ATPase 4
BA	Benzyl adenine
bp	Base pair
Cd	Cadmium
DDD	1,1-Dichloro-2,2,-bischlorophenylethane
DDE	dichlorodiphenylchloroethylene
DDT	1,1,1-trichloro-2,2-bischlorophenylethane
DNA	Deoxyribonucleic acid
EDTA	Athyldiaminetetraacetic acid
FAO	Food and Drugs Organization
Fe	ferros
GA_3	Gibberellic acid
H_2SO_4	Sulfuric acid
$HClO_4$	Perchloric acid
HCl	Hydrochloric acid
IAA	Indol 3-acetic acid
IBA	Indol 3-butyric acid
Kin	kinetin
LSD	Least significant difference
MAPK	Mitogen activated protein kinases
mM	Milli Molar

MS	Murashige and Skoog medium
NAA	Naphthaleneacetic acid
NaOH	Sodium hypochlorite
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
ppm	Part per million
TBE	Tris-borate EDTA
Zn	Zinc

CHAPTER ONE

Introduction And Literature Review

1. Introduction and Literature review

1.1 Introduction

Ruta graveolens commonly known as rue or herb of grace is a species of *Ruta* grown as an herb. It is native to the Balkan Peninsular. Rue is cultivated as a medicinal herb being used to treat various diseases such as vitiligo, psoriasis, cutaneous lymphomas, used as an anti-HIV agent and shows anti-inflammatory, antimicrobial properties. It is also helps to protect against cell damage by free radicals and is used to treat varicose veins and as a traditional supplement to improve eyesight (Aljaiyash *et al.,* 2014). It is now grown throughout the world as an ornamental plant in gardens, especially because of its bluish leaves, and also sometimes for its tolerance to hot and dry soil conditions. (en.wikipedia.org)

Survival of plants under adverse environmental conditions relies on integration of stress adaptive metabolic and structural changes into endogenous developmental programs. Abiotic environmental factors such as drought and salinity are significant plant stressors with major impact on plant development and productivity thus causing serious agricultural yield losses. The complex regulatory processes of plant drought and salt adaptation involve control of water flux and cellular osmotic adjustment by osmoprotectants biosynthesis. Since salinity induced imbalance of cellular ion homeostasis, it is coped with regulated ion influx and efflux at the plasma membrane and vacuolar ion sequestration. Drought and salinity have additionally major detrimental impacts on the cellular energy supply and redox homeostasis that are balanced by global re-programming of plant primary metabolism and altered cellular architecture (Agarwal *et al.,* 2013).

Another abiotic stress is the presence of heavy metals. Some manmade activities like sewage sludge, fertilizers, and the discharge of domestic wastes in land were the main source of higher concentrations of these metals. They contaminate the environment by altering soil properties biomass, fertility, and crop yields and thus the human health (Varsha-Mudgal *et al.,* 2010). Heavy metals toxicity reflects its impact on plant growth including leaf chlorosis, a decrease in the rate of seed germination, and a crippled photosynthetic apparatus, often correlated with plant death. Contaminated soils pose a major environmental and human health concern; phytoremediation technology provides solution to this critical problem (Waoo *et al.,* 2014). Plant tolerance to environmental stresses is governed by an inter-related network of physiological and molecular mechanisms and understanding of these mechanisms and their genetic basis is an important aspect to develop plants as agents of phytoremediation (DalCorso *et al.,* 2008).

Thus, this project was designed to achieve the following purposes:

1. Investigating the ability of *Ruta graveolens* to tolerate heavy metals *in vitro.*
2. Developing a regenerated protocol of the plant.
3. Examination of regenerated plants for their tolerance to heavy metals.
4. Investigating the molecular bases of heavy metals tolerance in tissue culture.

1.2 Literature review

1.2.1 *Ruta graveolens*

1.2.1.1 General features

The common rue, *Ruta graveolens* L., also called "herb of grace", belongs to the Rutaceae family, order Sapindales, class Magnoliopsida, division Magnoliophyta. *Rue,* is an ornamental, aromatic, culinary and medicinal plant. The rue shrubs are 0.6-0.9 m tall, robust, semiwoody and perennial (Figure 1.1). It is also spread from around the Mediterranean Sea to Europe, North and South America and Southwest Asia. The preferred sites are warm, rocky areas with basic soils (Laura Burga *et al.,* 2005).

Rue contains more than 120 compounds of different classes of natural products such as alkaloids, coumarins, essential oils, flavonoids and furoquinolines (De Feo *et al.,* 2002). The medical properties of this plant are belonging to the presence of these biologically active compounds. Pharmacological effects of the plant extract include antimicrobial, antispasmodic, photosensitizing and an abortifacient (Aljaiyash *et al.,* 2014). Infusions and decoctions of aerial parts of rue were used as anti-inflammatory and anti-rheumatic medicine and for the treatment of hypertension, skin illness and rhinitis (Parajapati and Kumar, 2003).The antitumor activity of this plant has been scientifically proved on Dalton's lymphoma ascites (DLA), Ehrlich ascites carcinoma (EAC) and L929 cells (Preethi *et al.,* 2006).

Figure 1.1 *Ruta graveolens* L. grown in a garden (internet)

1.2.1.2 *Ruta graveolens* tissue culture studies

Wide applications of *R. graveolens* L. in pharmaceutical industry has led to increased interest in large-scale plant production, with emphasis on use of *in vitro* cultures (Diwan and Malpathak, 2008). A number of workers have attempted to multiply *R. graveolens* using different explants. Kuzovkina (1980) obtained complete plant from stem explants. Castro and Barros (1997) developed protocols for micropropagation of the species from shoot tip, inter nodal and leaf segments and evaluated different culture media for optimum micropropagation .

Direct shoot bud induction in nodal segments of *R. graveolens* through axillary shoot multiplication has been reported by Faisal *et al.,* (2005). Bohidhar *et al*. (2008) reported highly efficient and cost effective protocol for high frequency plantlet regeneration from nodal explants, and study the effect of plant growth regulators. A protocol for multiple shoot bud induction and plant regeneration from leaf segment-derived callus of has been developed by Ahmed *et al.* (2010).

However, Tejavathi and Manjula (2010) represent a study on the effect of a wide range of plant growth regulators and amino acids on callus induction and multiple shoot regeneration from the cultures of *R. graveolens*, by using direct and indirect regeneration methods. Callus induction and whole plant regeneration protocol was represented by Zuraida *et al.* (2014), who exploited *R. graveolens* stem segments as explants.

In nature *R. graveolens* is propagated by seeds or through vegetative methods, but but these methods of propagation cannot meet the requirement, as the number of plants produced is limited. Propagation through seeds is hampered by low germination rate and low viability (Bohaider *et al.,* 2008).

1.2.2 Phytoremediation

Phytoremediation is one of the promising methods for reclamation of soils contaminated with toxic metals by using hyperaccumulator plants (Ghosh and Singh, 2005; Lazaro *et al.,* 2006). More than 400 plant species belonging to 45 plant families have been identified and reported from temperate to tropical regions with the ability to tolerate and hyperaccumulate trace elements (Baker and Brooks, 1989). These plants have been considered suitable for soil stabilization and extraction of heavy metals. Hyperaccumulator plants can play a key role in the fate of the pollutants of contaminated matrixes by their root systems. (Madejon *et al.,* 2002).

Phytoremediation is advantageous because it is more economically viable using the same tools and supplies as agriculture; it is less disruptive to the environment and does not involve waiting for new plant communities to recolonize the site; disposal sites are not needed; it is more likely to be accepted by the public as it is more aesthetically pleasing than traditional methods; it avoids excavation and transport of polluted media thus reducing the risk of spreading the contamination, finally, it has the potential to treat polluted sites with more than one type of pollutants (Hegedusova *et al.,* 2009).

Disadvantages of phytoremediation are that the limited tolerance of the plants, particularly encountered at high concentration of contaminants, lower efficiency over other non-biological remediation techniques and the limitations when the contaminated soil layer occasionally extends to the deeper profile (Wei *et al.,* 2004; Wu *et al.,* 2006). Another major issue is the handling and disposal of the

contaminated plant waste which could be land filled, composted or incinerated (Keller *et al.,* 2005; Rathinasabapathi *et al.,* 2006).

Conventional technical decontamination techniques are too costly to remediate extended areas of contaminated soils, and are also environmentally destructive. These remediation strategies are an *ex situ* approach and can be damaging to soil structure, ecology and productivity. As an alternative, plant-based bioremediation technologies have received attention as strategies to clean up metal-contaminated soil and water (Jadia and Fulekar 2008, Mary *et al.* 2012).

All plants have the ability to accumulate from soil and water metals such as Fe, Mn, Zn, Ni, Mo, Mg, and Cu, which are essential for their growth and development. Besides this, they can also absorb Cd, Pb, Co, Cr, Ag, Se, and Hg which have no biological importance in their physiological activities. Aliyu and Adamu (2014) showed that maize can be used to phytoremediate Fe, Zn, Mn, and Pb.

1.2.2.1 Phytoextraction

Phytoextraction of metal-contaminated soil relies on the use of plants to extract and translocate metals to their harvestable parts. The aim of phytoextraction is reducing the concentration of metals in contaminated soils to regulatory levels within a reasonable time frame. This extraction process depends on the ability of selected plants to grow and accumulate metals under the specific climatic and soil conditions of the site being remediated. Two approaches have currently been used to reach this goal: the use of plants with exceptional, natural metal accumulating capacity, the so-called *hyperaccumulators*, and the utilization of high-biomass crop plants, such as corn, barley, peas, oats, rice, and Indian mustard with a chemically enhanced method of phytoextraction (Chen *et al.,* 2004).

1.2.2.2 Phyto/Rhizofilteration

It is the use of plant roots to absorb and accumulate toxic metals from contaminated soil or water. Once the roots are saturated they are harvested, minimizing disturbance. Plants that are efficient in translocation metals to the shoots are generally not used. Rhizofilteration is relatively inexpensive and more effective than technological methods (Yadav *et al.,* 2011).

1.2.2.3 Phytostabilization

This involves absorption and precipitation of contaminants, especially metals, by plants, reducing their mobility and preventing their migration to ground water or to wind transport. The aim of phytostabilization is not to remove the metal pollutants from the site but to stabilize them, in order to reduce the risk to living organisms by entry into the food chain (Madejon *et al.,* 2009; Andreazza *et al.,* 2011).

Actually, there is three mechanisms within phytostabilization that determine the fate of the contaminants are: Phytostabilization in the root zone (immobilization of contaminants in the root zone due to exudations of the roots in the rhizosphere); Phytostabilization of root membrane, by binding of contaminants to root membrane (surface),thereby not allowing it to enter the plant; and Phytostabilization in the root cells (if contaminants are transported they can be sequestered into the vacuole of root cells preventing translocation) (Shilev *et al.,* 2009).

1.2.2.4 Phytodegradation

This is a breakdown of organic contaminants existing within or outside plants, with the help of enzymes produced by plants. Pollutants are degraded into simpler substances which are taken up by the plant to help them grow faster. Plants which produce enzymes that metabolize

contaminants may be released into the rhizosphere, where they can remain active in contaminant transformation. Enzymes such as peroxidase and nutrilase have been discovered in plant sediments and soils (Schnoor *et al.,* 1995). Uptake and degradation of DDT (dichloro diphenyl trichloro ethane) by *Brassica juncea* and *Cichorium intybus*, and phytotransformation of DDT to its main detoxiied metabolites DDD and DDE by hairy root cultures, has been reported (Suresh *et al.,* 2005).

1.2.2.5 Pytovolatilization

This is the process where plants take up contaminants which are water-soluble and release them into the atmosphere as they transpire the water. The contaminant may become modified along the way as the water travels along the plant's vascular system from the roots to the leaves. The contaminants may then evaporate or volatilize into the air surrounding the plant.

Experimental investigations have shown that volatile compounds are released to the atmosphere in significant amounts where plants are grown in soil containing these compounds (Marr *et al.,* 2006; Banuelos and Lin, 2007; Nwoko, 2010). Indian mustard has the ability to remove selenium by volatilization (Pilon-Smits *et al.,* 2010).

1.2.2.6 Zinc phytoremediation

Zinc is the second most abundant transition metal in organisms after iron (Fe), and the only metal presented in all six enzyme classes, oxidoreductases, transferases, hydrolases, lyases, and isomerases (Broadley *et al.,* 2007). Though Zn toxicity in crops is far less widespread than Zn deficiency, Zn toxicity occurs in soils contaminated by mining and smelting activities, in agricultural soils treated with sewage sludge, and in urban soils enriched by anthropogenic inputs of Zn (Chaney *et al.,* 1997)

Phytoextraction, using metal accumulating plants to transport and concentrate heavy metals from the soil into their harvestable biomass, has been proposed as an environmentally friendly and low-input remediation technique (Mcgrath and Zhao, 2003) .The discovery of zinc accumulation in certain *Viola* and *Thlaspi* species in the nineteenth century was followed by other species with that able to accumulate more than 10,000 $mg.kg^{-1}$ Zn accumulations, such as *Arabidopsis halleri*. (Roosens *et al.,* 2008).

1.2.2.7 Iron phytoremediation

Iron is a biogenic element present in plants in low quantities. It is polyvalent and has an effect on numerous physiological and biochemical processes. It has the capacity to form chelates, which are complex compounds of metals with certain organic compounds. Plants absorb iron from the soil in the form of ferrous ions (Fe^{2+}), ferric ions (Fe^{3+}), and in the form of iron chelates. Fe^{+2} is physiologically active. The high pH value, high concentration of phosphates and calcium ions influence the reduced uptake of iron. Iron plays a very important role in biosynthesis of chlorophyll, the first phases of photosynthesis, respiration, fixation of elemental nitrogen, reduction of nitrates and nitrites and metabolism of carbohydrates. It also affects cell division, elongation of plants, and root growth. Iron contents in dry matter of plants have wide range from 50 to 1000 μg/g (Briat *et al.* 2006)

Some plants are able to accumulate iron in significantly greater quantities so that for example leaves of spinach contains up to 3000 μg Fe g^{-1} of dry matter. Distribution of iron within plants is very characteristic. In the aboveground plant parts, it is the most present in leaves then in stem and grain. Plants take out of the soil more Fe than any other microelement through their crops. In case of surplus of Fe the growth of

all vegetative organs is inhibited. Leaves become dark to blue-greenish, root gains umber color. The symptoms of surplus of Fe are usually followed by the signs of deficiency of phosphorous and manganes. Two herbs that appeared as hyperaccumulators of iron: hoary plantain *Plantago lanceolata* that able to accumulate 2765 μg/g, and common dandelion *Taraxacum officinale* that accumulates 2425 μg/g (Stankovic *et al.,* 2011).

1.2.2.8 Cadmium phytoremediation

Cadmium is a nonessential heavy metal widespread in our environment because of contamination by power stations, metal industries, and waste incineration. Toxicity to living cells occurs at very low concentrations, with suspected carcinogenic effects in humans. However, the biological effects of this metal and the mechanisms of its toxicity are not yet clearly understood (Suzuki *et al.,* 2001).

Uptake of Cd by plant roots depends on the concentration, the oxidation state of this metal in solution, and on the physical–chemical characteristics of the soils such as pH content of clay, minerals, and organic matter (Gao *et al.,* 2011). Few plant species have shown to accumulate more than 100 mg kg^{-1} into their tissue (*Thalaspi caerulescens* and *Arabidopsis halleri,* both Brassicaceae) (Tolra *et al.,* 2006; Liu *et al.,* 2008). Recently, high accumulation abilities by Salix (Salicaceae) were shown (Tlustos *et al.,* 2007).

1.2.3 Biotic stress

Plants are under constant assault by biotic agents, including viral, bacterial and fungal pathogens, parasitic plants and insect herbivores, with enormous economic and ecological impact. These disease factors cause electrolyte leakage, changes in ion fluxes, cell death and other

stress responses, underlining the similarities in plant responses to microbial necrotrophy and abiotic stresses (Abu-Qamar *et al.,* 2008).

Once an attack is perceived, plant metabolism must balance potentially competing demands for resources to support defense versus requirements for cellular maintenance, growth and reproduction (Berger *et al.,* 2007).

In vitro selection is an alternative approach for development of stress tolerant lines. It is an important tool for desirable plant selection through enhanced expression of pathogenesis-related proteins, antifungal peptides or biosynthesis of phytoalexins (Ganesan and Jayabalan, 2006)

This technology is easy and cost effective compared to the transgenic approach for the improved disease tolerance). *In vitro* selection for resistance to a pathogen can be carried out using organogenic or embryogenic calli, shoots, somatic embryos or cell suspensions by exposing them to toxins produced by the pathogen, pathogen culture filtrate or to the pathogen itself (Kumar *et al.,* 2008).

1.2.4 Abiotic stresses

Abiotic stresses which include salinity, drought, high or low temperatures, light; deficient or excess nutrients, heavy metals and pollutants are commonly cause of plant loss worldwide. The stress caused by abiotic factors alter plant metabolism leading to negative effects on growth, development and productivity of plants. If the stress become harsh and continues for longer period it may lead to unbearable metabolic burden on cells leading to reduced growth and, in extreme cases results in plant death (Rao *et al.,* 2006). Plant adaptation to environmental stresses is controlled by cascades of molecular networks. These activate stress responsive mechanisms to re-establish homeostasis and to protect and repair damaged proteins and membranes (Wang *et al.,* 2012).

A cell is separated from its surrounding environment by a physical barrier, which is the plasma membrane. The cellular responses to a biotic stresses are initiated primarily by interaction of the extracellular material with a plasma membrane protein. This extracellular molecule is called a ligand or an elicitor and the plasma membrane protein, which binds and interacts with this molecule, is called a receptor. Various stress signals both abiotic as well as biotic serve as elicitors for the plant cell (Mahajan and Tuteja, 2005).

Tolerance to a biotic stresses is very complex at the whole plant and cellular levels (Munns and Tester 2008, Grewal 2010). Successful application of biotechnology to abiotic constraints being faced by plants will require knowledge of good biological information regarding the target species as well as the mechanisms underlying resistance/tolerance to these stresses (Dita *et al.,* 2006).

The plant responses to stress are dependent on the tissue or organ affected by the stress. For example, transcriptional responses to stress are tissue or cell specific in roots and are quite different depending on the stress involved (Dinneny *et al.,* 2008). In addition, the level and duration of stress can have a significant effect on the complexity of the response (Tattersall *et al.,* 2007). Plants show a great ability to adapt their metabolism to rapid changes in the environment. For this purpose they are equipped with complex processes, such as perception transduction and transmission of stress Stimuli (Devato *et al.,* 2005).

In contrast to plant resistance to biotic stresses, which is mostly dependent on monogenic traits, the genetically complex responses to abiotic stresses are multigenic, and thus more difficult to control and engineer (Vinocur and Altman, 2005). The conventional breeding programs are being used to integrate genes of interest from inter crossing

genera and species into the crops to induce stress tolerance. However, in many cases, these conventional breeding methods have failed to provide desirable results (Rai, 2011).

1.2.4.1 Salt stresses

Soil salinity is a major environmental stress, which threatens global food security. Up to 20% of the world's irrigated land, which produces one third of the world's food, is salt affected (FAO, 2011). Soil salinity existed long before humans and agriculture but the problem has been aggravated by agricultural practices such as irrigation and poor drainage systems. It has been predicted that increasing salinization in agricultural fields will reduce the land available for cultivation by 30% within the next 25 years, and up to 50% by the year 2050 (Wang *et al.,* 2003).

Plants differ greatly in their growth response to saline conditions and therefore classified as glycophytes or halophytes referring to their capacity to grow on highly saline environments. Some plants have evolved and adapted to freshwater habitat for acquiring nutrients from the low concentrations of minerals present in fresh water such as glycophytes, whereas the plants which retained their habitat in nutrient-rich marine environment were found more successful to combat the adverse abiotic stresses and are referred as halophytes (Flowers *et al.,* 2010).

Excess salt in soil or in solutions interferes with several physiological and biochemical processes that result in many problems such as ion imbalance, mineral deficiency, osmotic stress, ion toxicity and oxidative stress; these ultimately interact with several cellular components, including DNA, proteins, lipids, and pigments in plants (Zhu, 2002).

Therefore plant regulates the expression of salt-response proteins to reestablish cellular balance to reduce or adapt salt stress. As a result, plant

changed in development, morphology, and physiology. The deleterious effects of salinity on plant growth are associated with low osmotic potential of soil solution, nutritional imbalance, specific ion effect, or a combination of these factors biochemistry (Kamal *et al.,* 2010).

1.2.4.2 Water stresses

A water stress can be defined as the situation when the plant cannot cope anymore with a soil water deficit, leading to a decrease of water content in the tissues, and thus importantly modifying its metabolism. Plant responses to water stress can be studied *in vitro* but also under controlled conditions, by water withholding or by using osmotica such as polyethylene glycol (PEG), mannitol or melibiose. Benefits and drawbacks of each of these experimental methods are presented by Verslues *et al.,* (2006).

The cellular water deficits results in the concentration of solutes, loss of turgor, change in cell volume, disruption of water potential gradients, change in membrane integrity, denaturation of proteins and several physiological and molecular components (Bartels and Souer, 2003). The stress effects depend on the degree and duration of the stress, developmental stage of the plant, genotypic capacity of species and environmental interactions. Several attempts were made to understand the water stress recognition and the subsequent signal transduction (Mori and Schroeder, 2004).

1.2.4.3 Heavy metal stresses

Heavy metal (HM) ions play essential roles in many physiological processes. The term (HM) includes only elements with specific gravity above five but frequently biologist's use this term for a vast range of metals and metalloids which are toxic to plants such as copper (Cu), iron (Fe), manganese (Mn), zinc (Zn), nickel (Ni), cobalt (Co), cadmium (Cd),

and arsenic (As). Importantly, few HMs and transition metals such as sodium (Na), potassium (K), calcium (Ca), magnesium (Mg), Fe, Cu, Zn, Co, or Ni are, at certain concentrations, essential micronutrients that are critically involved in the functional activities of large numbers of proteins involved in sustaining growth and development of living organisms. However, problems arise when cells are confronted with an excess of these vital ions or with non-nutritional ions that lead to cellular damage (Gaetke and Chow, 2003).

In the course of industrialization, emissions of metals have been tremendously raised and significantly exceed those from natural sources for practically all metals. Due to this mobilization of metals into the biosphere, their circulation through soil, water, and air has greatly increased (Boran and Altinok 2010).

Although HMs are natural constituents of soils and occur naturally in the environment, nowadays, contamination of soils by toxic metals and metalloids is of major concern worldwide and become one of the main abiotic stress agents for living organisms. The problem of HM pollution is continuously worsening due to a series of human activities, leading to an intensification of research dealing with the phytotoxicity of these contaminants and with the mechanisms used by plants to counter their detrimental effects (Rascio and Navari-Izzo, 2011).

1.2.4.3.1 Heavy metal impact on plant

The effect of their toxic influence on plants is largely a strong and fast inhibition of growth processes of the above- and underground parts, as well as the activity decrease of the photosynthetic apparatus, often correlated with progressing senescence processes (Alaoui-Sosse *et al.,* 2004)

Depending on their oxidation states, heavy metals can be highly reactive, resulting in toxicity in most organisms. The ability of plants to increase antioxidative protection to combat negative consequences of heavy metal stress appears to be limited, as it has been shown that exposure to elevated concentrations of redox reactive metals results in decreased rather than increased activities of antioxidative enzymes (Mazars *et al.,* 2010).

Generally, the toxic effects of heavy metals on plants can be characterized by production of reactive oxygen species by autoxidation and the Fenton reaction, which is typical for transition metals such as iron and copper; blocking of essential functional groups in biomolecules, which has been mainly reported for non-redox-reactive heavy metals such as cadmium and mercury; displacement of essential metal ions from biomolecules, which occurs with different kind of heavy metals (Douchiche *et al.,* 2010).

However, as different HMs have different sites of action within the plant, the overall visual toxic response differs between HMs. The most widespread visual evidence of HM toxicity is a reduction in plant growth (Sharma and Dubey, 2007) including leaf chlorosis, necrosis, turgor loss, a decrease in the rate of seed germination, and a crippled photosynthetic apparatus, often correlated with plant death. All these effects are related to ultrastructural, biochemical, and molecular changes in plant tissues and cells in the presence of HMs (Gamalero *et al.,* 2009).

1.2.4.3.2 Heavy metal tolerance mechanisms

Plants have many detoxification and tolerance mechanisms that enable them to survive in a polluted soil containing toxic levels of heavy metal/metals. These mechanisms include establishment of symbiotic associations with soil microorganisms such as mycorrhiza that restrict

movement of heavy metal ions and uptake by the plant (Arriagada *et al.,* 2009). Immobilization of metals in the fungal biomass is proposed as a mechanism by which these fungi may increase plant tolerance to heavy metals. Mycorrhizal roots may act as a barrier against metal transport, reducing transfer and enhancing root/shoot Cd ratios (Andrade and Silveira, 2008).

Binding to the cell walls and eventually to root exudates, in fact, is the first line of defense against heavy metals; plant roots secrete exudates into the soil matrix. One of the major roles of root exudates is to chelate metals and to prevent their uptake inside the cells. It has been reported that root exudation of citrate maize in response to Cd stress contributing to Cd resistance (Colzi *et al.,* 2011)

Reduced influx through the plasma membrane, the plasma membrane plays an important role in plant response to heavy metals by preventing or reducing the uptake of metals into the cell or by active efflux pumping outside the cell (Lang and Wernitznig 2011).

Chelation in the cytosol by various ligands such as phytochelatins and metallothioneins and further heavy metals compartmentalization in vacuole is potentially a very important mechanism of HM detoxification and tolerance in plants under HM stress (Hasan *et al.,* 2009). One such strategy consists of transporting the HM out of the cell or sequestrating it into the vacuole, thereby removing it from the cytosol or other cellular compartments where sensitive metabolic activities takes place (DalCorso *et al.,* 2008)

Action of heat shock proteins (HSPs) connected with the stress caused by heavy metals. It not act as molecular chaperones in normal protein folding and assembly just, but may also function in the protection and repair of protein under stress conditions. Induction of HSPs by Zn and Cd

has been reported by Zhen *et al.* (2007). Increased accumulation of a large HSP (HSP70) was reported in response to Cd stress (Sarry *et al.* 2006).

1.2.5 *In vitro* selection of salt and drought tolerant plants

In recent years, tissue culture based *in vitro* selection has emerged as a feasible and cost-effective tool for developing stress-tolerant plants. Plants tolerant to both the biotic and the abiotic stresses can be acquired by applying the selecting agents such as NaCl for salt tolerance and PEG or mannitol for drought tolerance in the culture media (Rai *et al.,* 2011). Development of abiotic stress tolerant plants especially for salt and drought conditions using *in vitro* selection has been reported in a wide range of plant species including cereals, vegetables, fruits and other commercially important plant species (Manoj *et al.,* 2011)

Development of stress tolerant plants through *in vitro* selection has some limitations like loss of regeneration ability during selection, lack of correlation between the mechanisms of tolerance operating in cultured cell, tissue or organ and those of the whole plants, and phenomenon of epigenetic adaptation. Numerous studies reported that altered DNA methylation is a major cause of epigenetic modifications, which are sometimes observed in tissue cultured. Many authors suggested that the problem of epigenetic adaptation during *in vitro* selection can be overcome by the use of short term or one-step selection that may prevent the development of epigenetically adapted (Gao *et al.,* 2010).

1.2.6 *In vitro* selection of heavy metals tolerant plants

Plants have developed their own specific responses against both of biotic and abiotic stresses as well as cross-stress responses. Investigating

these responses is difficult under field conditions, but plant tissue culture techniques are performed under aseptic and controlled environmental conditions for the purpose of both commercial, like mass production, and scientific studies like germplasm preservation, plant breeding, physiological, and genetic (Rao *et al.,* 2006).

These advantages of plant tissue culture allow various opportunities for researcher to study the unique and complex responses of plants against environmental stresses (Lokhande *et al.,* 2011), and to study the physiological and biochemical changes in both unorganized cellular (i.e. suspension cultures and callus cultures) and organized tissue (i.e. axillary shoot, shoot tip, mature embryo, whole plant) levels (Patada *et al.,* 2012). Additionally, plant tissue culture techniques also allow opportunities for the researcher to improve plants against abiotic stress factors with the *in vitro* selection method (Jain, 2001).

Tissue culture technique was utilized by Verret *et al.* (2004) and showed that overexpretion of AtHMA4 will enhance heavy metals shoot to tip translocation. Seed derived calli of rice cultivars, IR72 and C14-8, were screened in vitro to study Fe toxicity and profiled for isozymes, esterase, peroxidase, malate dehydrogenase, and lactate dehydrogenase, to assess their involvement in Fe toxicity tolerance (Roy and Mandal, 2005). Waoo *et al.* (2014) represented a study describes standard protocol for in vitro production of plantlets of *Datura inoxia* and *Lantana camara*, the plants having phytoremediation potential and used to study the accumulation and tolerance mechanism of heavy metals cobalt (Co) and lead (Pb).

1.2.7 Cross talk in response to biotic and abiotic stresses

1.2.7.1 Reactive oxygen species

ROS have inevitably been factors for aerobic life since the introduction of molecular oxygen (O_2) into the atmosphere by O_2-evolving photosynthetic organisms. ROS can simply be described highly reactive and partially reduced-oxygen such as the superoxide radical ($O_2^{\cdot -}$), singlet oxygen ($1O_2$), hydroxyl radical ($OH^{\cdot}$), hydroperoxyl radical ($HO_2^{\cdot}$), hydrogen peroxide (H_2O_2). They are produced during metabolic pathway in several compartments of plants, including chloroplasts, mitochondria, peroxisomes, plasma membrane, apoplast, endoplasmic reticulum, and cell-wall, and also as a result of induced environmental stress factors (Mittler *et al.,* 2004).

When exposing to environmental stress factors, ROS levels can dramatically increase and this increase, in the later stage, leads to oxidative stress. Oxidative stress is defined a serious imbalance between the production of ROS and antioxidant defense and this situation can cause damage to cellular macromolecules, including proteins, lipids, carbohydrates and DNA (Gill and Tuteja, 2010).

ROS are scavenged by various antioxidant defense systems. Generally, antioxidant defense systems categorized into two groups, enzymatic antioxidants (superoxide dismutase, SOD; catalase, CAT; ascorbate peroxidase, APX; glutathione reductase, GR; monodehydroascorbate reductase, MDHAR; dehydroascorbate reductase, DHAR; glutathione peroxidase, GPX; guaiacol peroxidase, POX and glutathione-S- transferase, GST), and non-enzymatic (ascorbate, glutathione, carotenoids, phenolic compounds, proline, glycine betain, sugar, and polyamines) defense systems (Ahmad *et al.,* 2008).

Hoffmann (2010) represented a study on *R. graveolens* and exhibited that its help to protect against cell damage by free radicals and is used to treat varicose veins and as a traditional supplement to improve eyesight. Gurudeeban *et al*. (2011) developed a protocol for *in vitro* propagation and evaluated antioxidant and anti aggregating properties of micro propagated leaf and callus extract of *R. graveolens*. The enzymatic antioxidants catalase, super oxide dismutase, glutathaione reductase, glutathaione peroxidase and glutathiaone-S-transferase; and non enzymic antioxidants ascorbic acid, α- tocoperol, reduced glutathiaone, total carotenoids and flavonoids were found to be present. The free radicals scavenging (DPPH, hydroxyl radical and nitric oxide) activity was also observed.

1.2.7.2 Phytohormone ethylene

The gaseous phytohormone ethylene is a key regulator in plant growth and developmental process as well as biotic and abiotic stress response (Li and Guo, 2007). Despite its structural simplicity, ethylene is participates in many aspects of plant developmental processes, including seed germination, cell elongation, fruit ripening, organ senescence, root nodulation, programmed cell death, abscission and response to environmental stress, and pathogen attack (Bleecker and Kende 2000).

A relatively simple metabolic pathway controls the biosynthesis of ethylene. The biological precursor of ethylene, Methionine, is converted to S-adenosylmethionine (SAM) by SAM Synthetase. 1-aminocyclopropane-1-carboxylic acid (ACC) Synthase (ACS) uses SAM as a substrate to form ACC. This is mostly the rate-limiting step in the biosynthesis of ethylene. ACC is oxidised to ethylene by ACC Oxidase (ACO), with CO2 and cyanide as by-products (Vandenbussche *et al.,*

2012). Plants exposed to toxic levels of Cd, Cu, Fe, and Zn produce higher levels of ethylene by upregulating ACC synthase expression and activity (Maksymiec, 2007). Rodriguez-Serrano *et al.* (2009) revealed that pea plants accumulated a remarkable amount of ethylene in leaves when exposed to Cd for 14 days. ACS2 gene expression was also upregulated by high salinity (Achard *et al.,* 2006).The necrotrophic fungus *Botrytis cinerea* is known to induce ethylene production through an ACS2 and ACS6 dependent mechanism (Li *et al.,* 2012). Cadmium induced the biosynthesis of ACC and ethylene in Arabidopsis thaliana plants mainly via the increased expression of ACS2 and ACS6 which confirmed in the acs2-1acs6-1 double knockout mutants, (Schellingen *et al.,* 2014).

There is evidence that ethylene influence root system architecture RSA. Gallie *et al.* (2009) examined the tissue-specific expression of both ACS and ACC oxidase (ACO) genes in the elongating root of maize and concluded that ethylene production was limiting for root elongation. (Thomann *et al.* 2009) revealed that mutation in ACC synthase 5 (ACS5) will up-regulate ethylene biosynthesis in Arabidopsis which resulted in repression of primary root elongation. The role of ethylene in regulating RSA, and how this is mediated via auxin, is also well established. It is clear that ethylene is stimulating auxin biosynthesis in the root tip, where the higher concentration may inhibit growth (Strader *et al.,* 2010).

CHAPTER TWO

Materials 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
Atomic absorption spectrophotometer 5000	Perkin-Elmar, USA
Autoclave	Karl, Spain
Centrifuge	Hettch, USA
Culture vessels	Slamid, Germany
Distillator	GFL, Germany
Electric balance	Scaltec, Switzerland
Electric microwave oven	Grant, England
Gel electrophoresis system	Biocom, UK
Hot plate with magnetic stirrer	Gallenkamp, England
Laminar air flow cabinet	Al-Shaddad, Iraq
Micropipettes	Eppendrof, Germany
Nano-drop spectrophotometer	Thermo, USA
pH- meter	Toledo, England

Refrigerator	Ishtar, Iraq
Sensitive balance	Delta Range, Switzerland
Thermal cycler Agilent Sure Cycler 8800	Agilent, Germany
Vortex mixer	Heidolph, Germany
Water bath	GFL, Germany

2.1.2 Chemicals

The following chemicals were used during this study:

Chemicals	Company and origin
Ammonium nitrate, Potassium nitrate, Calcium chloride anhydrate, Magnesium sulphate anhydrate, Potassium phosphate monobasic, Boric acid, Potassium iodide, Manganese sulphate. $4H_2O$, Zinc sulphate. $7H_2O$, Molybdic acid. $2H_2O$, Cupric sulphate. $5H_2O$, Cobalt chloride. $6H_2O$, Sodium ethylene diamine tetraacetate, Ferrous sulfate. $7H_2O$, Thiamine. HCl, Nicotinic acid (free acid), Pyrodoxine. HCl, Glycine, Kin, NAA, BAP, 2,4-D, GA_3, IBA, Citric acid, Na_2HPO_4, Phosphoric acid, Sulfosalicylic acid, and Tris base.	BDH-England

Ethanol absolute 99.5%, Methanol, Glucose, NaOH, HCl.	Euroclone-Italy
Agarose, TBE, and loading buffer, Plant DNA Polymerase (2.5 U/µl), dNTP Mix (dATP, dCTP, dGTP, dTTP) 0.2 mM each, Plant PCR Enhancer, $MgCl_2$, Plant-DNA extraction buffer(Tris-HCl, EDTA, 2% ß-mercaptoethanol, ethidium bromide, DNA ladder.	KAPA3G Biosystems - USA
Specific primers pairs Pp400 and Pp600	Promega- USA

2.2 Methods

This work was carried out in the plant tissue culture Lab., Biotechnology Dept., Al-Nahrain University, during the period 7/10/2013-1\11\2014.

2.2.1 Sterilization of equipments

Glasswares were scrubbed with brush in a detergent bath, and washed thoroughly with a tap water, placed in an oven for two hours till sterilization. Glasswares with discarded cultures, as well as contaminated ones, were autoclaved in order to liquefy the agar and kill any microorganism that may be present. They were then washed under tap water and autoclaved. Graduate cylinders, test tube, and flasks were plugged with absorbent cotton and aluminum foil in order to avoid any contaminants to enter them. Petri dishes were wrapped with aluminum

foil and placed inside the autoclave at 1.04 Kg.cm^2, 121°C, for 20 minutes and wet sterilized.

2.2.2 Plant material

Ruta graveolens plantlets were obtained from the Biotechnology Dept. College of Science, Al-Nahrain University, Iraq.

2.2.3 Sterilization of explants

R. graveolens internodal segments (about 1.0cm in length) were used as a source for explants. Stems were placed under tap water for 30 minutes, and then sterilized by continuous shaking with 2.0% v/v sodium hypochlorite (Clorox) for 20 minutes, followed by washing with sterilized distilled water three times. All the steps of sterilization were carried out under aseptic conditions using laminar air flow cabinet. Explants were transferred into sterilized Petri dishes having sterile filter papers to remove excess water (Gurudeeban *et al.,* 2011).

2.2.4 Preparation of plant tissue culture medium

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) components were prepared (table 2.1), supplemented with 30 g/l sucrose and growth regulators at different concentrations. The pH of the medium was adjusted to 5.8 using 1N NaOH or 1N HCl, then 7 g/l agar was added to the medium. The culture medium was autoclaved at a pressure of 1.04 kg/cm^2, 121°C, for 15 minutes, and then left at room temperature until use. The medium was dispensed into Petri dishes or 15x2.5cm vials (10 ml/tube).

Table 2.1 MS medium components used as stock solutions for plant tissue culture experiments.

Components	**Chemical formula**	**Weight (mg/l)**
Macronutrients		
Ammonium nitrate	NH_4NO_3	1650
Potassium nitrate	KNO_3	1900
Calcium chloride unhydrate	$CaCl_2.2H_2O$	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	$MnSO_4.4H_2O$	22.30
Zinc sulphate heptahydrate	$ZnSO_4.7H_2O$	8.60
Molybdic acid (sodium salt) dihydrate	$Na_2MoO_4.2H_2O$	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	Na2-EDTA	33.6
Ferrous sulphate heptahydrate	$FeSO_4.7H_2O$	27.8
Vitamins		
Thiamine.HCl	$C_{12}H_{17}CIN_4OS.HCl$	0.1
Nicotine acid (free acid)	$C_{18}H_{11}NO_3.HCl$	0.5
Pyridoxine.HCl	$C_6H_5NO_2$	0.5

2.2.5 Callus induction

The surface sterilized explants were cut into small segments, about 1 cm, and inoculated into callus induction medium, different combinations of various concentrations of 2,4-D (0, 0.75, 1.0 or 1.25 mg/l), and combined with kinetin (0, 0.75, 1.0 or 1.25 mg/l) were added. Cultures were incubated at 25±2°C in dark. Stem explants were cultured in each Petri dishes (5 per dishes) with 10 replicate for each treatment. After four weeks of incubation, callus induction frequency (%) was calculated using the following formula (Yousif, 2002).

Callus induction frequency (%) = No. of explants produced callus/total No. of cultured explants * 100

2.2.6 Maintenance of callus cultures

Small pieces of calli weighting 50 mg each were transferred into MS medium supplemented with 0.0, 1.0, 1.5 or 2.0 mg/l 2,4-D and combined with 0.0, 0.5, 1.0 or 1.5 mg/l Kin. Vigorous growing portions of calli were transferred while necrotic or brown calli were discarded before subculture on a fresh medium. Callus tissues were incubated as in 2.2.5. The experiment was carried out with 10 replicates for each treatment.

2.2.7 Measurement of callus fresh weight

Callus fresh weight was measured after four weeks of subculture under aseptic conditions.

2.2.8 Screening and Selection for drought and salinity tolerance

Callus pieces produced on stems explants weighting about 100 mg/tube were subcultured into MS medium supplemented with 1mg/l 2,4-D and 0.5 mg/l Kin and manitol at the concentration 300 mg/l to induce

drought stress or with saline water (drainage water) with an electrical conductivity (EC) 30ds.m^{-1} for inducing salt stress.

2.2.9 Determination of heavy metal concentrations

In order to determine the heavy metal concentrations used in this experiment, a sample of saline water (drainage water) was analyzed by using Atomic Absorption Spectrophotometer at IBN SINAA Company, Ministry of Industry and Minerals. Data (table 2.2) were compared with others of Baghdad drinking water (Barbooti *et al.,* 2010), and data from Diyala river (Easa *et al.,* 2011).

Table 2.2 Parameters measured in saline, drinking and Diyala river waters.

Parameter	Units	Saline water	Drinking water	Diyala river
Fe	mg/l	0.05	0.6	0.09
Cu	mg/l	0.001	< 0.005	0.09
Pb	mg/l	0.0005	0.02	0.003
Co	mg/l	0.002	-	-
Cd	mg/l	0.001	< 0.001	0.003
Zn	mg/l	0.012	0.02	0.06
EC	dS/m^{-1}	120	-	-
pH	-	8.8	7.05-7.85	

2.2.10 Screening and selection for heavy metal tolerance

Stock solutions (1000 ppm) of the metals Zn, Cd and Fe were used to prepare different concentrations of these metals (0.0, 0.1, 0.5, 1.0, 2.0, 3.0 ppm) to be added to the maintenance medium (MS supplemented with 1mg/l 2,4-D and 0.5 mg/l Kin). About 100 mg of salinity and drought tolerant calli were subcultured directly into previous prepared medium. Fresh weights were calculated after three weeks and the selected concentration of the heavy metals were determined. Callus cultures were re-cultured three times on the same medium. Each treatment was carried out with 10 replicates.

2.2.11 Determination of relative fresh weight and tolerance index

Relative fresh weight of the embryogenic calli was calculated at different stress levels according to the following formula (Daud *et al.,* 2014)

$$RFW = \frac{FW_F}{FW_I} \qquad (1)$$

where FWI = initial fresh weight and FWF = final fresh weight.

Fresh biomass-based tolerance index (TI) of *R. graveolens* callus culture was calculated according to the following formula:

$$TI\ (\%) = \frac{\text{Fresh biomass of stressed callus}}{\text{Fresh biomass of control callus}} \times 100 \qquad (2)$$

Since TI = Tolerance Index

Data were taken for four different stress periods at three week intervals, that is, 21, 42, and 63 days.

2.2.12 Shoot regeneration

Tolerant calli were selected and transferred into the regeneration medium under aseptic conditions. The regeneration medium consisted of half strength MS medium supplemented with BA (2.5 or 3.0 mg/l), NAA (0.5 mg/l). All cultures were maintained at 25±2 °C for 16/8 hrs (light/dark) photoperiod at a light intensity of 1000 lux.

2.2.13 Shoots elongation medium

All multiple shoots obtained were transferred to the same regeneration medium supplemented with 0.5 mg/l of GA_3 (Gurudeeban *et al.,* 2011).

2.2.14 Rooting medium

Shoots were transferred onto half strength MS medium supplemented with 0.1 mg/l IBA for rooting (Faisal *et al.,* 2005a; Ahmad *et al*., 2010).

2.2.15 Preparation of the samples

Plant leaves harvested from plants grown on a typical soil were harvested and subjected to analysis. Callus pieces grown in cultures supplemented with heavy metals were also harvested and dried at 60 °C for 24 hrs. and subjected to analysis. A quantity of 0.3 g of leaves or callus samples was placed in the digestion apparatus, then 10 ml of concentrated H_2SO_4 was added and heated till boiling for 2 hrs. until the color turned black. The solution was cooled, and 1.5 ml of $HClO_4$ was added, reheated until the solution became clear. The volume was completed to 50 ml using a volumetric flask. The concentrations of heavy metals in this solution were measured using Atomic Absorption Spectrophotometer (Bada and Raji, 2010).

2.2.16 Experimental design and statistical analysis

The experiments were designed as factorial experiments using a completely randomized design. Analyses were done using Genstat discovery version 4 software. Differences between means were determined and least significant differences were compared at $P \leq 0.05$ (Steel and Torri, 1982).

2.2.17 Molecular analysis of genomic DNA

2.2.17.1 Detection of ACC synthase gene in *Ruta graveolans*

In order to detect the presence of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase gene in *Ruta graveolans* plant in control and heavy metal treated plants, two locations on the sweet oranges (*Citrus sinensis*) ACC synthase gene were selected to design primes for PCR assay.

2.2.17.1.1 Specific primers

ACC synthase (ACS2) gene was selected to design two primer pairs for PCR assay. The primers were named Pp600 and Pp400, the sequence and nucleotide numbers were shown in table (2.3). Primers were provided in a lyophilized form and were dissolved in 10 mM Tris-HCl (pH 8.0 – 8.5) (Schori *et al.,* 2013).

Table 2.3 Specific primers for amplification of crude DNA of *R. graveolens*

Primer name	Forward (F) and reverse (R) primers sequence **5'------ 3'**		Product expected size (bp)
Pp600	F	GGGATCGAATTCAAGAGCGA	600
	R	AAGCCTGCAAACGTACGAAT	
Pp400	F	CGTACTCGTGCTGGTGGTAG	400
	R	CCAATTCATGGCAAAGCAGG	

2.2.18 Preparation of crude plant-DNA

For crude sample PCR with the KAPA3G Plant PCR Kit, crude extract of *R. graveolans* plant samples were prepared using the protocol outlined below.

2.2.18.1 Crude extract preparation protocol

Plant crude extraction was performed using the following protocol

1. Leaves pieces (~5 x 5 mm) of *R. graveolens* were cut using either a scalpel or a single-hole punch and placed into two microcentrifuge tubes for each sample.
2. Samples were crushed inside the 1.5 ml microcentrifuge tubes with sterilized stainless steel piston.
3. Aliquot of 100 µl of extraction buffer was added to each tube, to ensure that the entire sample is submerged in the buffer.
4. A sterile pipette tip was used to bruise leaf samples gently in the extraction buffer.
5. One of the two tubes from each sample was incubated at 95 °C for 5 minutes, once the incubation is complete; the tubes were placed on ice until PCR setup.

2.2.19 PCR optimization

Many experiments had been carried out in order to obtain optimum PCR results. These included manipulation in PCR conditions like denaturation and annealing temperatures, and PCR cycles number and duration till reaching the conditions outlined below.

2.2.19.1 Thermal cycling conditions

It was conducted according to the kit manufacturer's instructions and as in the following table. The thermal cycling device was Agilent SureCycler 8800 (Agilent, Germany).

Step	Temperature	Duration	No. cycles
Initial denaturation	95	3 min	1
Denaturation	95	20 sec	35
Annealing	58	15 sec	
Extension	72	15 sec/kb	
Final extension	72	2 min/kb	1

2.2.19.1 PCR components

Each reaction was set up as follows**:**

Component	Concentration	Amount (µl)
KAPA Plant PCR Buffer	2X	25.0
$MgCl_2$	25 mM	1.0
Forward Primer	10 µM	1.5
Reverse Primer	10 µM	1.5
KAPA Plant PCR Enhancer	100X	1.0
KAPA3G Plant DNA Polymerase	2.5 U/µl	0.5
Plant Crude extract	---------	1.0
PCR-grade water	---------	18.5
Total volume		50.0

2.2.20 Gel electrophoresis

2.2.20.1 Preparation of 1.0% agarose gel

It was prepared as follows:

1. Agarose (1g) was added to 100 ml of TBE 1X buffer, placed in a microwave for 1.5 min until the agarose granules were completely melted and the solution looks clear.
2. Ethidium bromide (2 µl) was added to the agarose solution after cooling down to 50-60 ºC.
3. The solution was then poured into a tray, the comb was inserted and the agarose gel was allowed to solidify for about 30 min.
4. The electrophoresis apparatus was assembled and plugged in a direct current, agarose gel tray was placed into a tank, filled with 1X TBE buffer sufficient to cover the entire gel.
5. The comb was removed carefully, and then each well was loaded with DNA or PCR product.

2.2.21 Analysis of PCR products

PCR products and 100 bp DNA ladder were determined by electrophoresis. Aliquot of 3 µl of loading dye plus 7µl of the product were mixed and loaded on 1.0% agarose gel and run at 100 volt for 40 sec. Bands were visualized on UV transsiluminator and then photographed.

2.2.22 Field experiment

Well-developed shoot with root systems were removed from the culture, washed carefully with tap water and transferred to pots containing sterilized soil. The plantlets were hardened for 10 to 15 days in green house, and then placed in the normal environment. Heavy metals tolerance was investigated by culturing *R. graveolens* plantlets in

soils contaminated with Zn and Cd at concentrations 10 and 1 mg/kg soils respectively. Experiment was achieved in pots with a diameter of 16 cm using clay silt soil containing: sand (140) g/kg, clay (640) g/kg, and silt (220) g/kg. Each pot contains 2 Kg of soil that contaminated with heavy metals. These pots were put under field conditions.

ChapterThree

Results and Discussion

3. Results and Discussion

3.1 Callus induction

3.1.1 Effect of 2,4-D and kinetin on callus induction

An experiment was carried out to induce callus on intermodal segment explants. After four days of culturing on MS medium, explants showed an elongation reponse, three weeks later, callus induction was observed at cutting edges then covering the entire explants surface after four weeks. The resulted callus appeared green watery texture.

Callus induction percentage was also assessed (table 3.1). Addition of kin increased the % of callus induction at the concentration 1.0 and 0.75 mg/l significantly recording 75.24 and 55.02% compared with 0.0 mg/l kin (32.72%). However, the % of callus induction reduced significantly (16.14%) when the kin concentration was increased to 1.25 mg/l.

All 2,4-D concentrations (0.75, 1.00, 1.25 mg/l) led to a significant increase in the % of callus induction which gave 41.47, 59.12 and 56.14% while the control treatment (0.0 mg/l) recorded 22.39%. The maximum % of callus induction occurred when 1.0 mg/l of 2,4-D was interacted with 1.0 mg/l of kin achieving 100%.

Data presented in table 3.1 were similar to those obtained by Ahmad *et al.* (2010) who reported light yellow friable callus induction in *R. graveolens* using MS medium supplemented with different concentrations of 2,4-D, and showed that the frequency of callus production increased with increasing of 2,4-D concentration , recording 45% at 0.5 mg/l and 68.3% at 2 mg/l 2,4-D. Gurudeeban *et al.* (2011) reported that

maximum % of callus induction was achieved using MS medium enriched with 0.5 mg/L IAA and 1.0 mg/l 2,4-D which yielded compact yellowish callus. At low concentration of IAA and 2,4-D (0.5 and 0.25 mg/L respectively) little response was noticed. Best frequency of compact yellowish green callus at 0.5 mg/L of IAA and 1.0 mg of 2,4-D.

Table 3.1 Effect of 2,4-D and kin and their interaction on the % callus induction, after inoculating explants on MS medium for six weeks, n=10.

Kin (mg/l)	2,4-D (mg/l)				mean
	0.00	0.75	1.0	1.25	
0.00	0.00	32.58	44.00	54.33	32.72
0.75	33.45	55.67	70.33	60.63	55.02
1.00	43.64	64.33	100.0	93.00	75.24
1.25	12.47	13.32	22.18	16.62	16.14
mean	22.39	41.47	59.12	56.14	
LSD 0.05	2,4-D= 7.2; Kin= 6.8 Interaction= 13.4				

The trend of these results are in accordance with those reported by Shah *et al.* (2003) who stated that a medium supplemented with relatively high 2,4-D concentrations induce good callus growth and proliferation of wheat cultures. According to Rashid *et al.* (2009), the addition of 2 mg/L of 2,4-D was the most effective for callus induction where 97.18% callus induction was recorded in *Triticum aestivum* cv. tatara. Hussain *et al.* (2010) reported the same conclusion in rice tissue cultures.

Results were also in line with the work of Hakkima *et al.* (2011) who reported that The highest % callus induction was observed in the *Ocimum sanctum* (L.) cultures supplemented with 2,4-D (1 mg/L) and Kin (0.1 mg/l), and with Bano *et al.* (2005) work, who reported that excellent rice (*Oryza sativa* cv. Swat-II) callus resulted on MS medium supplemented with 2,4-D and kin. Dalila *et al.* (2013) indicated that 2,4-D and kinetin at certain combinations in MS medium gave better callusing compared to other basal media for inducing callus for somatic embryogenesis production of *Barringtonia racemosa*. Results were also in agreement at Karimi *et al.* (2014) who obtained 70% callus induction on the medium containing 1 mg/l Kin+1 mg /l 2,4-D in *Saturejaa vromanica*.

Considering the stimulatory effect of 2,4-D on plant growth and its basic role in cell elongation, a significant effect of 2,4-D was confirmed in increasing callus formation. Kin also increased callus production, but increasing its concentration caused a considerable decrease in callogenesis. This shows the negative effect of increasing the concentration of cytokinin in callus formation. Small concentrations of 2,4-D and kinetin enhanced mitosis, but increased concentrations had inhibitive effect. Auxin had aneugenic potential by acting as spindle poison and disturbing the correct separation of the chromosomes at cell poles (Truta *et al.,* 2011)

3.2 Maintenance of callus cultures

In order to maintain and increase callus mass, portions of calli obtained from previous experiment were transferred to MS medium supplemented with different concentrations of plant growth regulators (table 3.2). Mean callus fresh weight recorded 254.40 mg at 0.0 mg/l kin

then increased at 0.5 mg/l reached 481.823 mg and reduced at 1.0 mg/l and 1.5 mg/l reached 420.367 and 61.073 mg respectively.

Mean callus fresh weight was highest at 1.0 mg/l 2,4-D reached 338.678 mg compared to concentrations 1.5 mg/l and 2.0 mg/l recorded 289.308 and 278.513 mg respectively. The interaction between treatments showed that the highest callus fresh weight was recorded at 1.0 mg/l 2,4-D and 0.5 mg/l kin reached 524.44 mg. Callus fresh weight decreased when both 2,4-D and kin concentrations were increased reached its lowest value at 2.0 mg/l 2,4-D and 1.5 mg/l kin recorded 44.36 mg.

Table 3.2 Effect of 2,4-D and kinetin and their interaction on mean callus fresh weight (mg), after inoculating callus pieces on solid MS medium for four weeks. Initial weight was 150 mg, n=10

Kin (mg/l)	2,4-D (mg/l)			mean
	1.0	1.5	2.0	
0.0	230.50	202.10	303.60	245.40
0.5	524.44	455.71	465.32	481.823
1.0	517.40	443.2	300.50	420.367
1.5	82.37	56.22	44.63	61.073
mean	338.678	289.308	278.513	
LSD 0.05	2,4-D=32.6 ;Kin=28.2 Interaction=61.3			

Results showed in table (3.2) are in agreement with those of Rashid *et al.* (2009) who reported that kinetin and increasing 2,4-D concentrations up to 3 mg/L and 4 mg/L affect callus fresh weight negatively in *Triticum aestivum* cv. Manthar. Results are also in line with Ilahi *et al.*

(2005) work who induced embryogenic callus on rice (*Oryza sativa* L. CV SWAT II) cultures in MS medium containing 1.0 mg/l 2,4-D and 0.5 mg/l kin. Among the various types of auxins and cytokinins, 2,4-D initiates callus along with the addition of Kin at concentrations 3 mg/l 2,4-D and 3 mg/l kin on *Dolichandrone falcate* explants (Bansode *et al.* 2013).

It is well documented that auxins promote cell division particularly in the presence the cytokinins (Skoog and Miller, 1957). The auxin 2,4-D found to be effective in callus induction at low concentrations. Auxins regulate cell elongation, tissue swelling, cell division, inhibition of adventitious and axillary shoot formation, and induction of embryogenesis. Kin at high concentrations has a negative effect on callus induction since it induces shoots rather than callus (Taiz and Zeiger, 1998).

3.3 Selections for drought and salt tolerance

Manitol at the concentration 300 mg/l was used to induce drought stress and saline water (drainage water) with an electrical conductivity (EC) 30 $ds.m^{-1}$ for inducing salt stress was selected according to Hadi *et al.* (2014), and then selected callus cultures were subjected to heavy metal stresses.

3.4 Effect of heavy metals on callus growth

Salinity and drought tolerant calli of *R. graveolens* were inoculated on MS medium containing different concentrations (0.0, 0.1, 0.5, 1.0, 2.0 or 3.0) of heavy metals (Zn, Cd, or Fe) to investigate the adverse effect on callus fresh weight.

3.4.1 Effect of zinc on callus relative fresh weight (RFW)

It's clear from the results shown in figure 3.1 that salinity tolerant calli, after three weeks of culturing, there is a linear increase in callus

RFW with the increasing of Zn at the concentrations 0.1, 0.5, and 1.0 ppm reached 1.82, 2.41, 2.81 g respectively. A decline in callus RFW occurred at the concentration 2.0 ppm recorded 1.26 g and further at 3.0 ppm, as the RFW reached minimum value (0.93 g).

While in case of drought tolerant calli (fig 3.2), there is a significant difference in RFW under Zn stress. Highest RFW was recorded at the concentration 0.5 ppm, reached 2.00 g, compared with the concentrations 0.0 and 0.1, which reached 1.62 and 1.73 g respectively. Means of callus fresh weight decreased at high concentrations 1.0, 2.0 and 3.0 ppm, recorded 1.58, 0.94 and 0.16 g respectively.

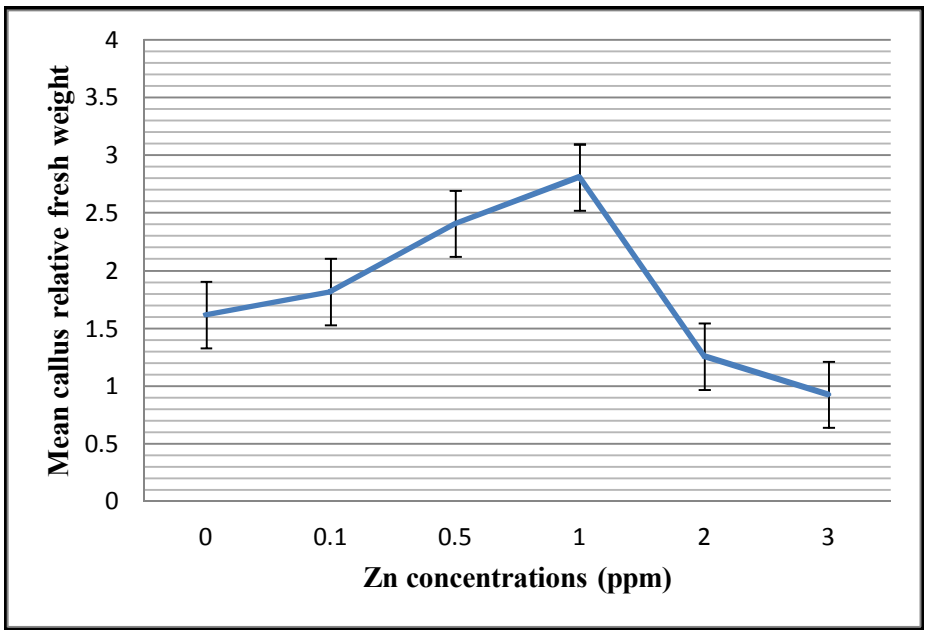

Figure 3.1 Callus relative growth curve for salt tolerant calli under Zn stress after three weeks. Bars represent standard errors.

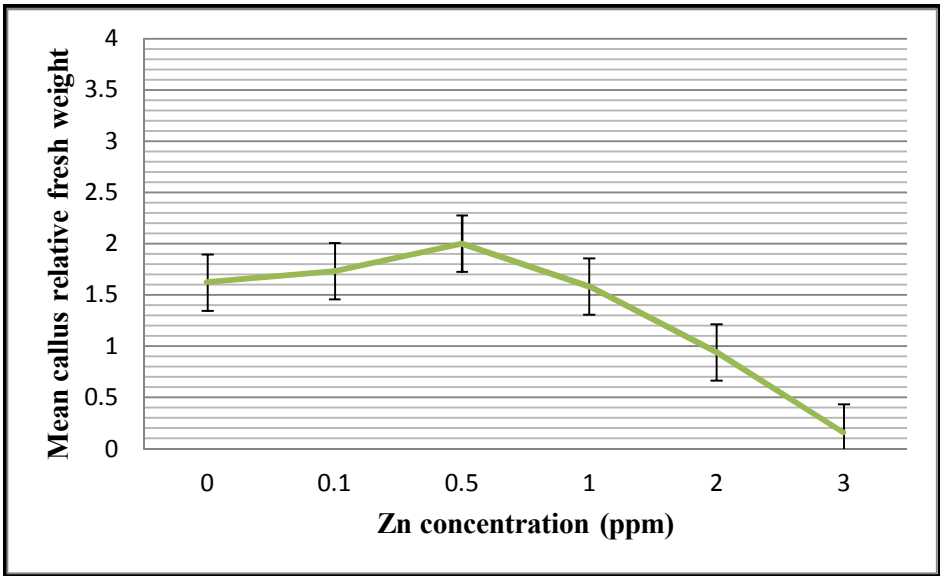

Figure 3.2 Callus relative growth curve for drought tolerant calli under Zn stress after three weeks. Bars represent standard errors.

Zinc is involved in many cellular functions such as protein metabolism, photosynthetic carbon metabolism and indole acetic acid metabolism, and the only metal presented in all six enzyme classes, oxidoreductases, transferases, hydrolases, lyases, and isomerases, yet its higher concentrations cause toxicity (Sinhal, 2007). Toxicity leads to an inhibition of cell activity or disruption of structures. High levels of Zn may stimulate the formation of free radicals; witch may lead to oxidative stress (Dietz *et al.,* 1999).

Results showed in figure 3.1 were in agreement with Luo *et al.* (2010) who studied the effects of different zinc concentrations on the growth and biomass of Jatropha seedlings and found that the biomass of cotyledons, hypocotyls and radicles increased gradually up to 0.25, 0.5 and 1.0 mM zinc and then decreased and development of cotyledons was inhibited, when zinc concentration was higher than 0.5 mM and toxic symptoms were observed on the cotyledons, hypocotyl and radicles. Yahya (2014)

reported there is a correlation between callus fresh weight and Zn concentration in callus cultures of *Prosopis farcta spp*., a linear increase in callus fresh weight with the increasing of zinc oxide nanoparticles concentration.

Results were also in line with Long *et al.* (2013) who studied three bacterial endophytes of *Sedum alfredii,* VI8L2, II8L4 and VI8R2, Results showed that three strains were re-introduced into *S. alfredii* rhizosphere soil under Zn stress and resulted in better plant growth, as roots biomass increased from 80% to 525% and shoot biomass from 11% to 47% compared with the un-inoculated ones.

3.4.2 Effect of cadmium on callus relative fresh weight (RFW)

In the current study, no differences between salinity tolerant and drought tolerant calli response to Cd stress in terms of fresh weight. Calli RFW values were very low at the high Cd concentration 3ppm reached 0.16 g in case of salinity tolerant calli (figure 3.3), and 0.14 g in case of drought tolerant calli (fig 3.4). While RFW at Cd concentration of 0.1 ppm was high in both cases, reached 2.05 and 2.03 g respectively. As showen in figure (3.3) and figure (3.4), increasing Cd concentration resulted in decreasing relative fresh weight. Moreover, data analysis showed that there is no significant differences in callus fresh weight.

Fornazier *et al.* (2002) showed that low concentration of $CdCl_2$ (0.1 mM) caused a significant increase in callus growth, whereas concentrations (0.5 and 1.0 mM) inhibited growth of callus culture in *Saccharum officinarum* callus cultures. Results are in agreement with Gomes-Junior et *al.* (2006), who demonstrated that at 0.05 mM $CdCl_2$, growth was stimulated in coffee suspension cells, but at 0.5 mM $CdCl_2$, the growth rate was reduced.

However, Shekhawat *et al.* (2010) showed a reduction in the fresh growth with the increasing concentration of Cd in calli of Brassica. Similar findings were reported by Sobkowiak and Deckert (2003) in soybean (*Glycine max*) cultures.

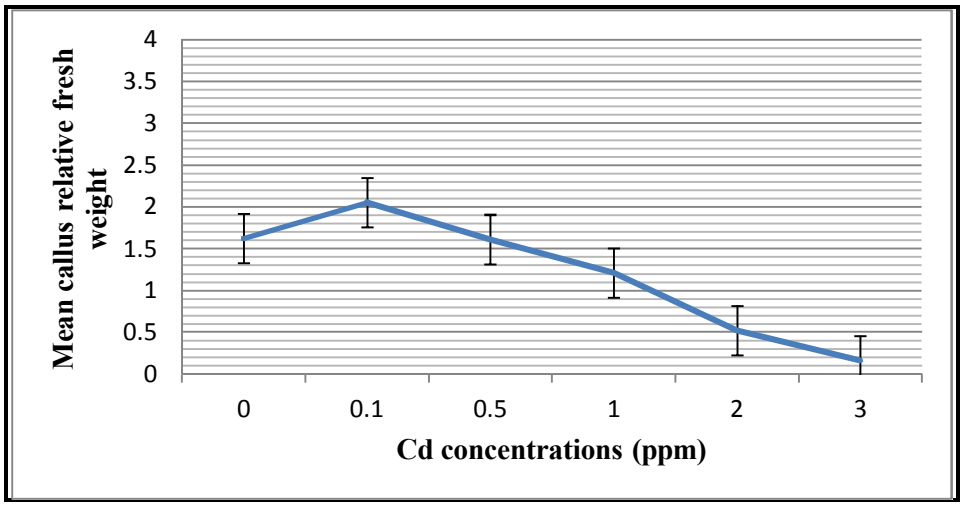

Figure 3.3 Callus relative growth curve for salt tolerant calli under Cd stress after three weeks. Bars represent standard errors.

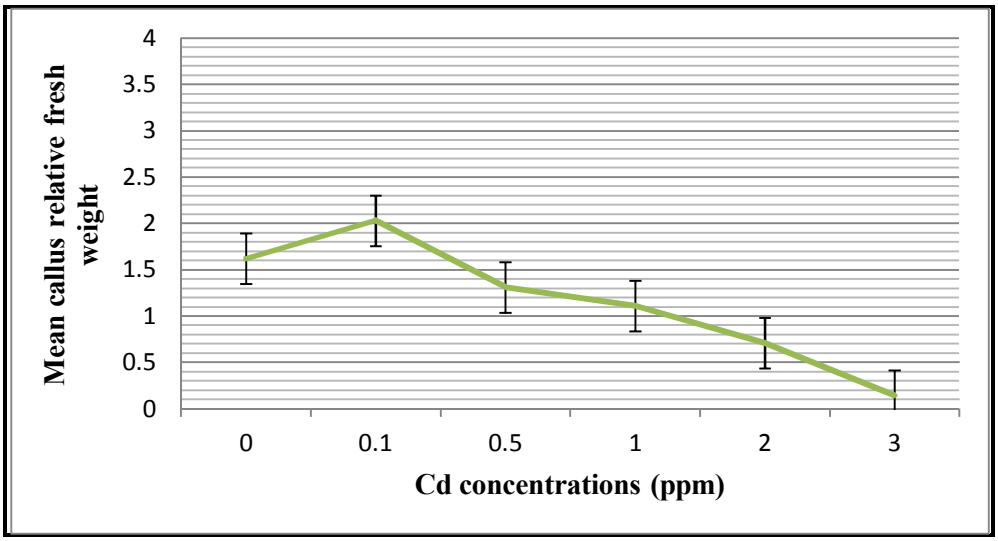

Figure 3.4 Callus relative growth curve for drought tolerant calli under Cd stress after three weeks. Bars represent standard errors.

Increasing in callus relative fresh weight at low Cd concentrations could be explained by competition between Zn or Fe and Cd for the same cellular binding sites since there is similarity in structural, geochemical, and environmental properties and can functionally substitute for Zn in plant cells. High concentrations of Cd result in toxicity represented by various functional-based alterations in plants such as growth retardation, changes in root morphology, root and leaf anatomy, and damages to cell structures as well as disturbance in water balance, mineral nutrition, photosynthesis, and plant development (Prasad, 1995).

3.4.3 Effect of different concentrations of iron on callus relative fresh weight (RFW)

Results displayed in figures (3.5) and (3.6) indicate that, in both salinity and drought tolerant calli, the highest value of RFW was recorded at 0.5 ppm Fe concentration, reached 3.58 and 4.15 g, then RFW values were decreased with increasing Fe concentrations and reached 0.35, 0.16 g at the concentration 3 ppm in salinity and drought tolerant calli respectively. Data analysis revealed that there is significant differences between Fe concentrations 0.1, 0.5, and 1.0 ppm compared to control in terms of fresh weight, while it did not recorded at concentrations 2.0 and 3.0 ppm. Results also exhibited the response of drought tolerant calli to different Fe concentrations and showed that there is excessive growth at low concentrations 0.1 and 0.5 ppm and the RFW reached 2.69 and 4.15 g respectively. Further increase in Fe concentrations 1.0, 2.0 and 3.0 ppm resulted in decreasing callus relative fresh weight, reached 1.17, 1.10 and 0.16 g respectively.

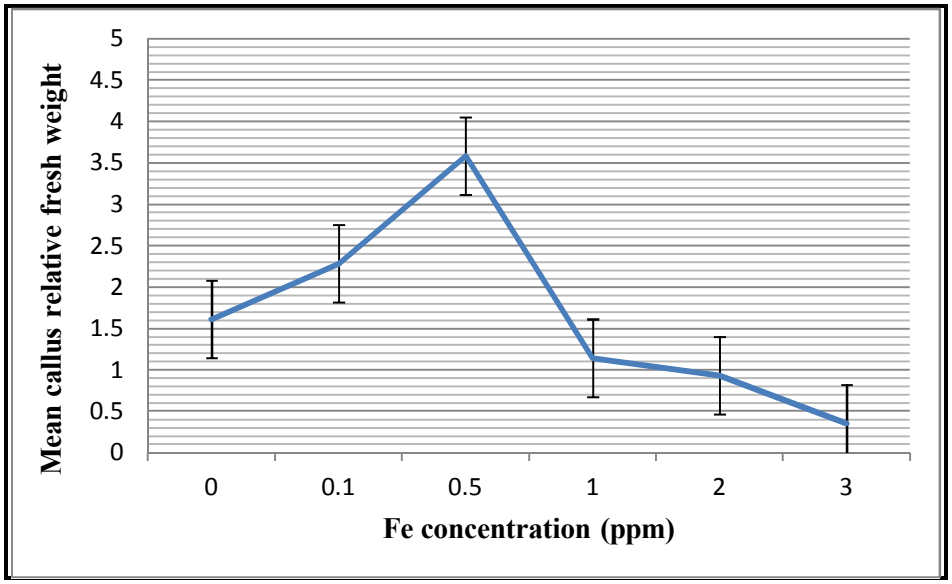

Figure 3.5 Callus relative growth curve for salt tolerant calli under Fe stress after three weeks. Bars represent standard errors.

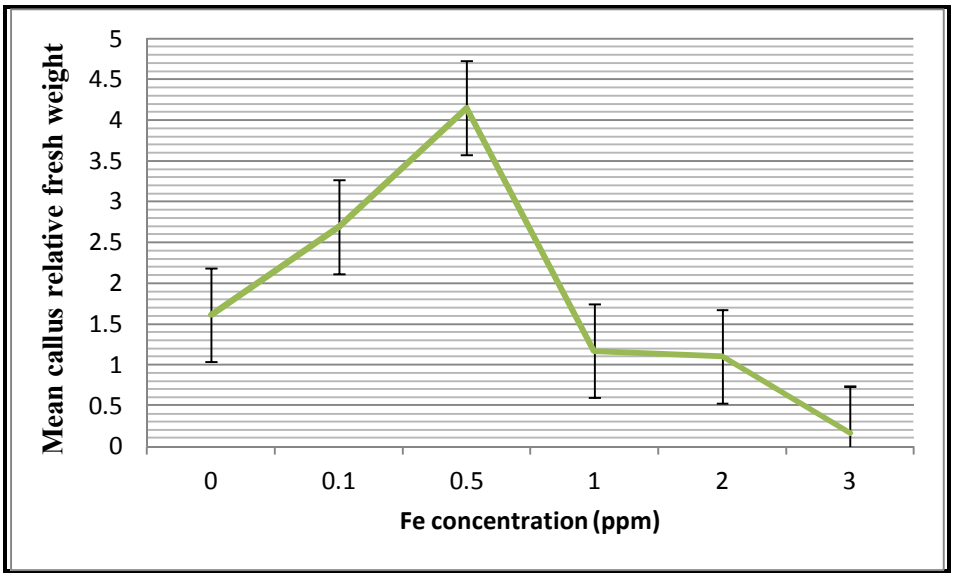

Figure 3.6 Callus relative growth curve for drought tolerant calli under Fe stress after three weeks. Bars represent standard errors.

Results come in line with Batty and Younger (2003) who studied the effect of different Fe concentrations on reed (*Phragmites australis*), Fe at

1 mg/ L was found to be optimum, above which growth of *P. australis* was significantly inhibited. Our findings were in agreement with Mandal *et al.* (2003), Roy and Mandal (2005) on rice that revealed the toxic effect of iron on plant at high concentrations.

Fe is an essential element in plants that is involved in many physiological processes, but that can also be toxic when provided in excess. In well-aerated soils, Fe is present as ferric hydroxides with low plant availability (Conte and Walker 2011). However, in anaerobic soils and at low redox potential, Fe is reduced to its soluble form Fe^{2+} and can be taken up excessively by plants. In plant tissues, Fe^{2+} participates in Fenton reactions, catalyzing the generation of hydroxyl radicals (OH) and other reactive oxygen species (Thongbai and Goodman 2000). These radicals oxidize chlorophyll and subsequently reduce leaf photosynthesis, thereby leading to yield reductions (Pereira *et al.,* 2013).

3.5 Tolerance index percentage

In order to study the dose and time-dependent effect of heavy metals on *R. graveolens* callus cultures, fresh biomass-based tolerance capability under various heavy metals stress levels for different duration was also investigated (Table 3.3). Data showed that, salt and drought tolerant calli, showed the highest tolerance index after 63 days under stresses of the heavy metals Zn, Cd, and Fe at the concentration 1 ppm, except in case of salt tolerant calli, as it showed its highest tolerance index value after 21 days under Cd stress at 1 ppm. Maximum values were recorded in salt tolerant calli reached 339.70 % under Zn stress, followed by drought tolerant calli under Fe stress reached 259.87%. Callus cultures under Cd stress showed less tolerance as the highest value was 149.89%.

Table 3.3 Tolerance index percentages (TI %) for *R. graveolens* callus cultures tolerant to salinity and drought then subjected to different concentrations of heavy metals after different time periods

Callus	Heavy Metal	Conc. (ppm)	Tolerance index (%) after		
			21 days	42 days	63 days
Non-stressed	_	0.00	0.00	00.00	0.00
Salinity tolerant	Zn	1.0	145.40	217.7	339.70
		2.0	86.20	75.00	74.00
		3.0	73.00	34.00	32.64
	Cd	1.0	84.35	71.87	67.77
		2.0	58.01	44.79	41.58
		3.0	44.27	34.37	19.75
	Fe	1.0	81.67	112.76	148.85
		2.0	73.66	92.18	64.65
		3.0	51.52	32.55	24.11
Drought tolerant	Zn	1.0	98.47	107.03	172.76
		2.0	74.04	62.76	54.67
		3.0	44.27	27.08	19.33
	Cd	1.0	80.05	106.25	149.89
		2.0	65.26	63.28	57.17
		3.0	43.51	27.08	19.12
	Fe	1.0	103.43	120.31	259.87
		2.0	80.15	99.47	75.59
		3.0	44.27	34.11	22.86

Tolerance capacity decreased at further increasing in heavy metals concentrations and exposure periods reached its minimum values at 3 ppm after 63 days in salt and drought tolerant calli. Its clear from these data that *R. graveolens* callus cultures were more tolerant to heavy metals at concentration 1 ppm and in the order Zn > Fe > Cd.

3.6 Shoots regeneration

Results shown in table 3.4 exhibited that half strength MS medium supplemented with BA at 2.5 mg/l gave the highest rate of shoot formation percentage reached 72% compared with 40% and 0% when the same medium supplemented with 2.5 mg/l BA plus 0.5 mg/l NAA and with 3.0 mg/l BA plus 0.5 mg/l NAA respectively.

Table 3.4 Effect of BA and NAA on % shoot formation after 8 weeks of culturing callus pieces on half strength MS medium. n=10.

BA(mg/l)	NAA(mg/l)	Shoot formation %
0.0	0.0	*
2.5	0.0	70
2.5	0.5	40
3.0	0.5	0
Mean		27. 5
LSD 0.05		5.40

However, BA at 2.5 mg/l induced the development of greenish nodular callus gave rise to minishoots (figure ٣.7 A) which later developed to shoots. The earliest sign of shoot formation and subsequent elongation was noticeable in un-stressed callus after 6 and 8 weeks of

incubation, respectively (figure 3.7 A), while tolerant callus took two weeks more (figure 3.7 B). Although the combination of 2.5 mg/l BA plus 0.5 mg/l NAA gave rise for high morphogenic capacity, a few numbers were developed to shoots later and the resulted shoots exhibited glassines appearance (figure 3.7C). Increasing BA to 3 mg/l resulted in inhibiting shoot induction and callusing of shoot bases was noticed (figure 3.7D).

These results come in line with those of Ahmad *et al.* (2010) who indicated that BA at 2.5 mg/l induced greenish nodular callus in *R. graveolens* cultures forming microshoots which later developed to shoots. Similar response was reported by Maity *et al.* (2005). Also in line with Tejavathi and Manjula (2010) who stated that the rate of shoots multiplication reduced with increasing cytokinin concentrations. Similar observations were made by Bohidhar *et al.* (2008) in the same taxon and was in agreement with the reports on *Coleus forskohlii* (Reddy *et al.,* 2001), *Tylophora indica* (Faisal *et al.,* 2005b).

On the other hand, these results disagree with the Ahmad *et al.* (2010) who studied the effect of adding low concentrations of NAA to the regeneration medium of the same taxon. Results also disagree with Tejavathi and Gayathramma, (2005) who stated that low auxin concentration in combination with a high cytokinin is the most suitable combination for the proliferation of shoots in *Agave vera-cruz* Mill.

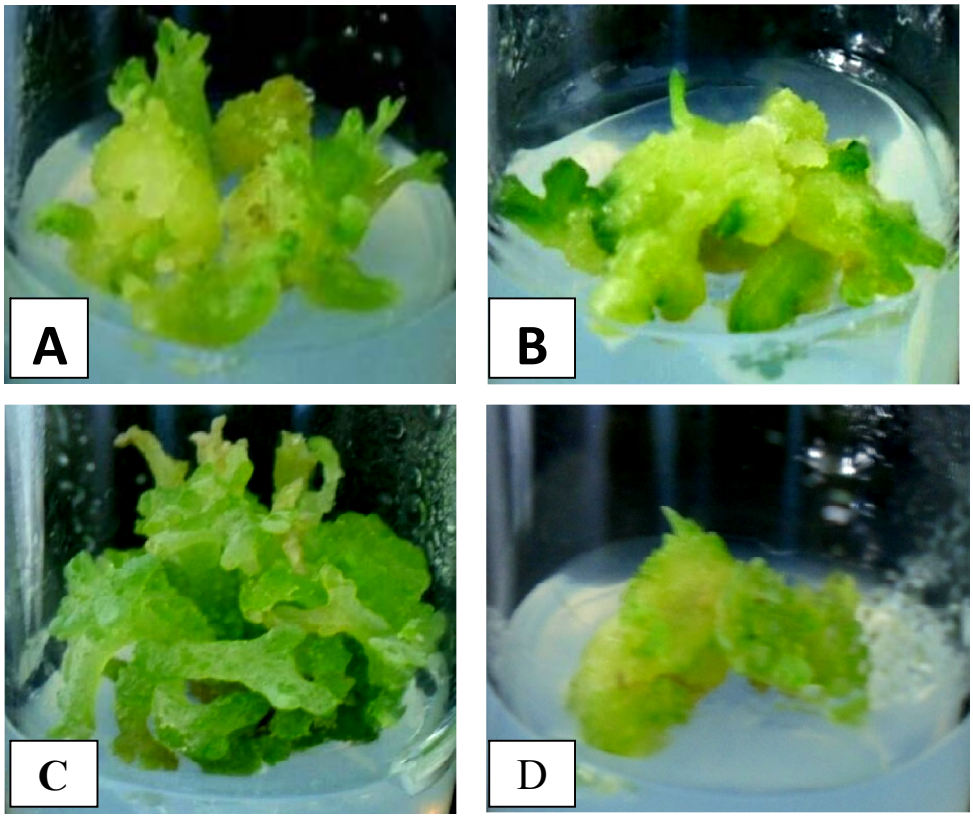

Figure 3.7 Regeneration of microshoots. A: un-stressed callus regenerated on half MS medium supplemented with 2.5 mg/l BA. B: shoots regenerated from tolerant callus on half MS medium supplemented with 2.5 mg/l BA. C: shoot induced on half MS medium supplemented with 2.5 mg/l BA and 0.5 mg/l NAA. D: shoots regenerated on half MS medium supplemented with 3.0 mg/l BA and 0.5 mg/l NAA.

BA, when added at suitable concentrations, it act by regulating cell division, stimulate auxiliary and adventitious shoot proliferation, regulate differentiation, inhibit root formation, activate RNA and protein synthesis and enzyme activity, delay senescence and promote nutrient uptake and cause greening process while NAA promote induction of somatic embryos only.

3.7 Shoots elongation and rooting

Single shoots (50-150 mm length) were transferred to the same regeneration medium supplemented with 0.5 mg/l GA_3. More shoots and longer were observed after two subculture cycles at two week intervals (fig. 3.8). Similar results were obtained by Gonbad *et al.* (2014) who supplemented different cytokinin concentrations combined with GA_3. They found that highest shoots number and longest shoots were obtained at combination of 3 mg/l BAP and 0.5 mg/l GA_3.

The outcome of the previous investigation was that the combination of BA and GA_3 could have two products simultaneously. The first product was the appropriate shoots for shoot multiplication by applying BA and the second one was shoots multiplication due to the use of GA3, which is proper for rooting.

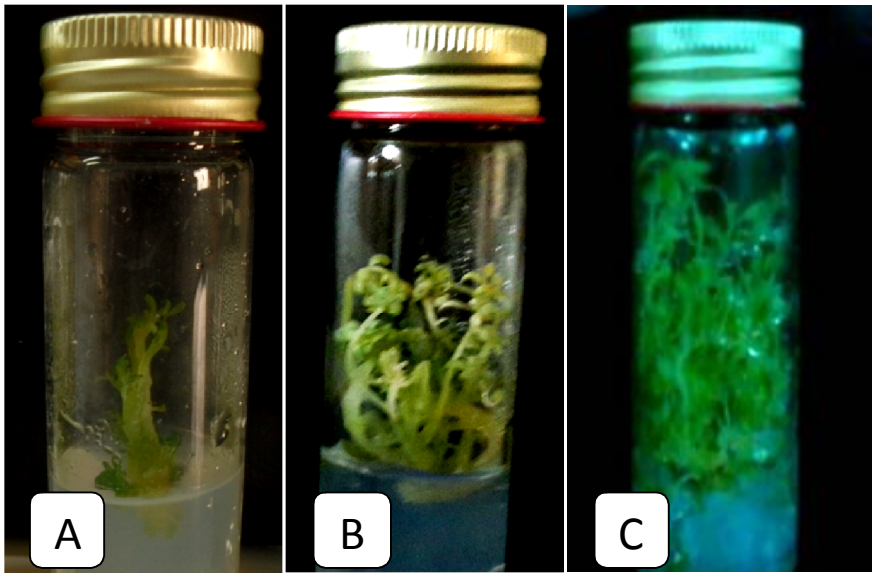

Figure 3.8 Shoot multiplication and elongation. A: shoots (50-150 mm length) were transferred to half strength MS medium supplemented with 2.5 mg/l BA and 0.5 mg/l GA_3 after two weeks. B: after four weeks. C: after six weeks.

Elongated and healthy shoots were transferred to a rooting medium consisted of half strength MS medium supplemented with 0.1 mg/l IBA (fig. 3.9). It is interesting that, un-stressed callus-derived plantlets showed early signs of root initiation than stressed callus- derived plantlets which took too long time for root induction (about eight weeks).

Batty and Younger (2003) stated a weak root growth, lack of branching of roots and root flaccidity in reed cultured under high Fe concentrations. Decreased roots number and length, and sharp depression in the mitotic activity of roots from sugarcane were reported by Jain (2010) under heavy metals stress.

Figure 3.9 Healthy elongated shoots cultured on half strength MS medium supplemented with 0.1 mg/l IBA for rooting.

3.8 Characterization of regenerated shoots

Noticeable morphological difference between shoots derived from non stressed calli and others derived from stressed calli after eight weeks. Longer shoots, a wide leaves surface area, typical leaves edges and more leaves number were recorded in non stressed shoots. While short shoots, small leaves surface area, and a sharp leaves edges were noticed in shoots derived from stressed callus (fig. 3.10).

Changes in leaves characteristics have been widely reported as a mechanism to cope with adverse environmental conditions. The phytotoxic effect of heavy metals in plants through visual symptoms such as reduced growth and biomass accumulation was also reported by Gomes *et al.* (2011). Gomes *et al.* (2013) stated that Cd induced the appearance of xeromorphic characteristics in leaves as decreased water potential, increased numbers and decreased stomata size in *Pfaffia glomerata* cultures.

This may be beyond the production of ethylene at high concentrations as a result of salt, drought and heavy metal stresses applied on plant *in vivo* which have adverse effect on plant in terms of growth and appearance. This was confirmed in the acs2-1acs6-1 double knockout mutants, which showed a decreased ethylene production, positively affecting leaf biomass.

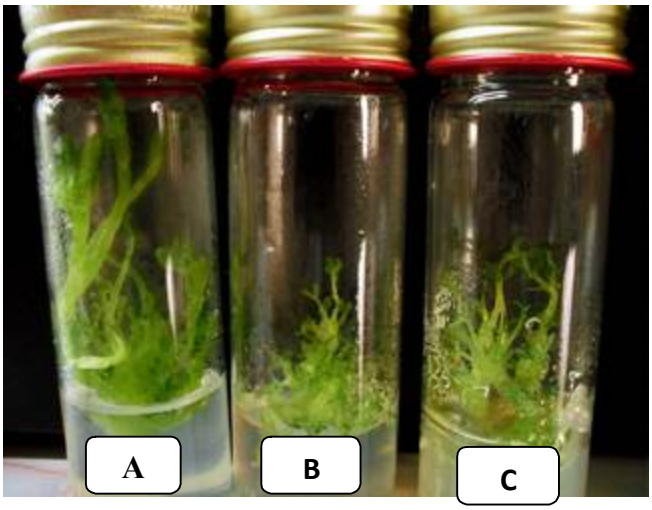

Figure 3.10 Morphological description of callus cultures differentiated to shoot. A: shoots derived from non stressed callus. B: shoots derived from callus under Fe stress. C: shoots derived from callus under Zn stress.

3.10 Quantitation of heavy metals

Heavy metals concentrations inside dried plant tissues were determined spectrophotometrically. As shown in table 3.5, *R. graveolens* found to accumulate high concentrations of heavy metals Zn, Fe or Cd in stressed callus reached 7100, 440 or 95 µg/g respectively compared to leaves grown in a typical soil reached 202, 310 or 3.00 µg/g respectively, and non-stressed callus reached 213, 177 or 0.00 µg/g respectively.

Table 3.5 A comparison between heavy metals content in leaves grown in a typical soil, non-stressed callus and stressed callus under heavy metals Zn, Cd and Fe at a concentration of 1 ppm.

Tissue type	Heavy metals concentration (µg/g)		
	Zn	Fe	Cd
leaves	202	310	30.0
Non-stressed callus	213	177	0.00
Stressed callus	7100	440	95

3.10 PCR assay

3.10.1 Detection of ACC synthase gene in *R. graveolens*

Results (figure 3.11; 3.12) exhibit that control sample produce a PCR product with a molecular size ~ 600 bp using Pp600 primers pair and a PCR product with a molecular size ~400 bp using Pp400 primers pair confirming the presence of ACC synthase gene (ACS2) in *R. graveolens.*

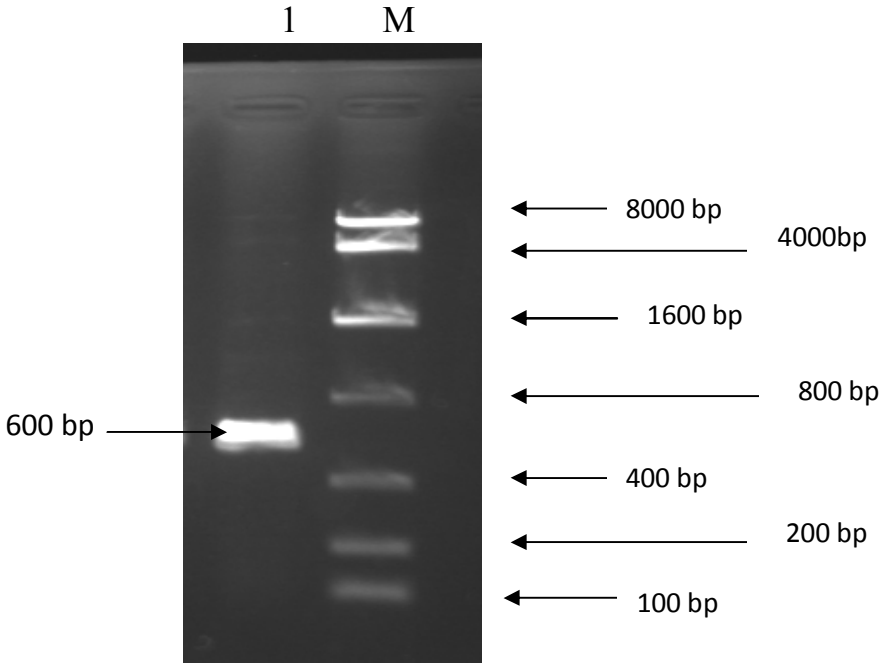

Figure (3. 11): Gel electrophoresis for ACS2 gene on agarose gel (1%) for 1 hour using specific primer Pp600

M: DNA ladder marker (100 bp).

Lane 1: ACS2 gene.

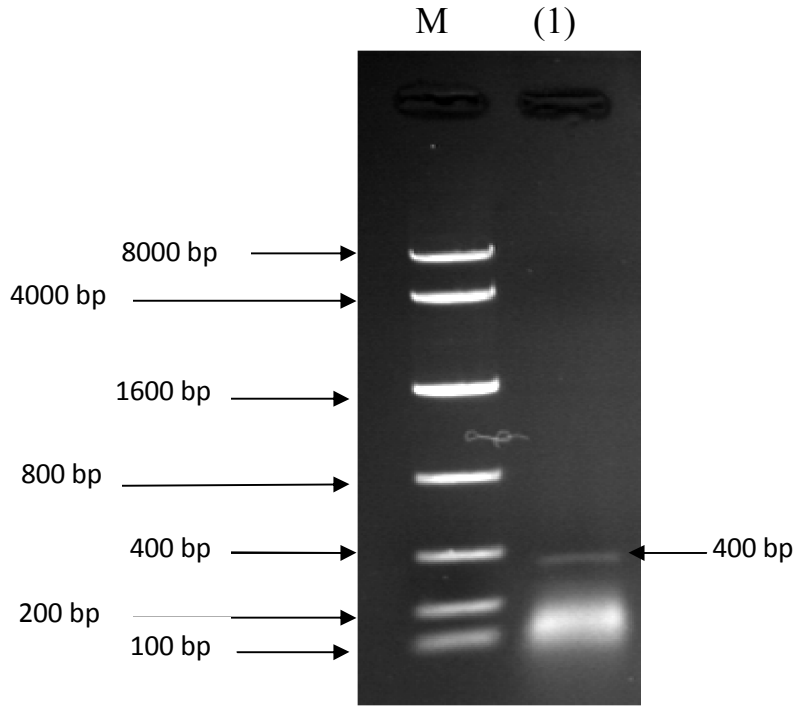

Figure (3. 12): Gel electrophoresis for ACS2 gene on agarose gel (1%) for 1 hour using specific primer Pp400

M: DNA ladder marker (100 bp).

Lane 1: ACS2 gene

Increased ethylene biosynthesis occurs in plants under a wide variety of stresses. The two key steps in ethylene biosynthesis are the synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) from S-adenosyl- L-Met, and the oxidative cleavage of ACC to form ethylene. The enzymes catalyzing these two reactions are ACC synthase (ACS) and ACC oxidase (ACO), respectively. Both enzymes are encoded by small gene families. (Schellingen *et al.,* 2014).

In general, the basal level activity of ACS is very low in tissues that do not produce a significant amount of ethylene. Stress-induced ethylene production is associated with a rapid increase in cellular ACS activity. By

contrast, ACO activity is constitutively present in most vegetative tissues. Therefore, ACS is the rate-limiting enzyme and the major regulatory step in stress-induced ethylene production (Chae *et al.,* 2003).

Actually, ACC is originating from a multigene family (ACS1-ACS12) which are regulated in a time-dependent manner or as required by physiological and developmental stages. Within this 12-membered family, ACS2, this is found to be activated after exposure to biotic and abiotic stresses (Marcos *et al.,* 2005; Peng *et al.,* 2005).

3.10.2 Effect of abiotic stresses on ACC synthase gene (ACS2)

Results shown in fig. (3.12) represent the effect of abiotic stresses salt, drought, and heavy metals on ACC synthase gene (ACS2). Results showed that all treatments produced a PCR product with a molecular size ~ 600 bp using specific primers pair Pp600. Results showed in fig. (3.13) indicated that *R. graveolens* produce a PCR products with variant molecular sizes using specific primers pair Pp400. Lanes 1,3 and 5 revealed that *R. graveolens* under drought and heavy metals (Fe, Zn, and Cd) stresses gave a product with a molecular size ~ 400 bp. lane 2, exhibited that *R. graveolens* under salt and heavy metals Fe produced a PCR product ~ 213 bp. lanes 4 represented *R. graveolens* under salt and Zn which produced a PCR product with a molecular size ~ 226 bp.

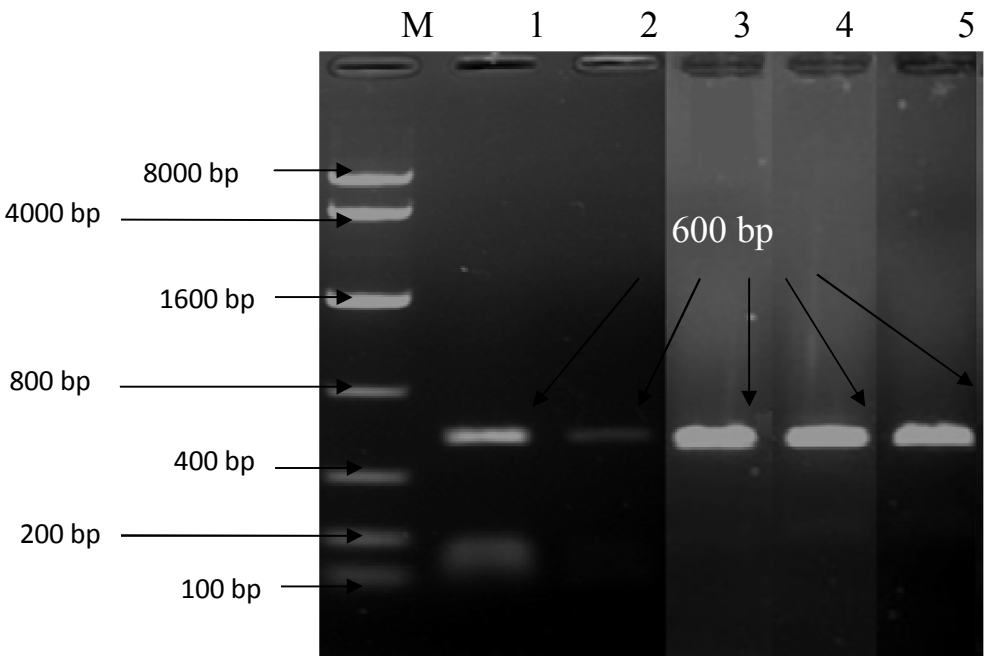

Figure 3.13 Agarose gel electrophoresis of PCR reaction using specific primer pairs Pp600. Band were fractioned using 1% agarose gel (1 hour, 100 v, 1X TBE buffer) and visualized under UV light. M: 100 bp ladder. Lane 1: Ruta under drought and Fe stresses. Lane 2: Ruta under salt and Fe stresses. Lane 3: Ruta under drought and Zn stresses. Lane 4: Ruta under salt and Zn stresses. Lane 5: Ruta under drought and Cd stresses.

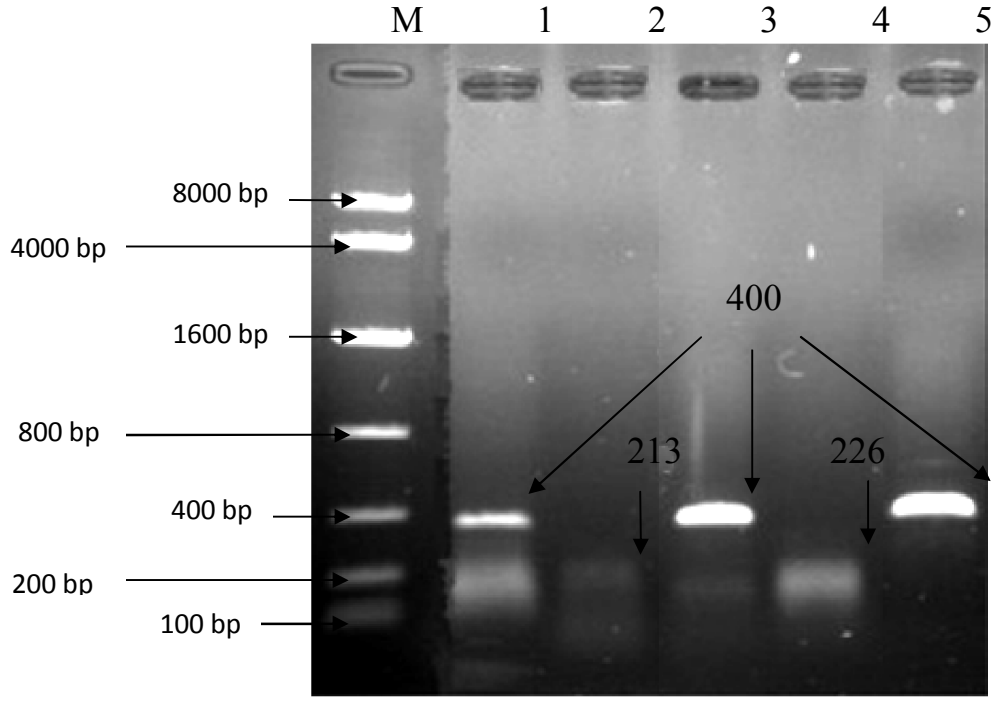

Figure 3.14 Agarose gel electrophoresis of PCR reaction using specific primer pairs Pp400. Band were fractioned using 1% agarose gel (1 hour, 100 v, 1X TBE buffer) and visualized under UV light. M: 100 bp ladder. Lane 1: Ruta under drought and Fe stresses. Lane 2: Ruta under salt and Fe stresses. Lane 3: Ruta under drought and Zn stresses. Lane 4: Ruta under salt and Zn stresses. Lane 5: Ruta under drought and Cd stresses.

The analysis of PCR amplified DNA fragments relies on the presence or absence of bands and on the differences in molecular weight regardless the intensity of amplified bands, as DNA concentration was not controlled by a specific concentration which mean that the number of DNA template copies was not the same in each sample that may affect the intensity of the resulted bands (Younan, 2010).

Elevated levels of cellular ACS activity and ethylene production under salt, drought, and heavy metal stresses were reported by many

authors who utilized different items genetically. Lin *et al.* (2008) reported functional characterization of transcription factor, Arabidopsis RAP2.4, in mediating light and ethylene signaling and found that expression of the RAP2.4 gene is down-regulated by light but up-regulated by salt and drought stresses. Zahir *et al.* (2009) reported that increased ACC activation and ethylene synthesis is accelerated in response to various environmental stresses like salinity. Maksymiec (2007) reported that plants exposed to toxic levels of Cd, Cu, Fe, and Zn produce higher levels of ethylene by upregulating ACC synthase expression and activity.

The current results showed that PCR analysis of *R. graveolens* samples subjected to salt and heavy metals stresses exhibit differences in molecular sizes of PCR products. This may be as a result of a mutation (deletion) in DNA. Mutation could be a result of many reasons, one of the most acceptable reasons is reactive oxygen species (ROS) which is reported by previous studies to be accumulated in plant tissues after exposure to salt (Miller *et al.,* 2010) and heavy metals which induce the generation of ROS and have to be kept under control because the presence of heavy metals in excess leading to excessive production of ROS. This causes cell death due to oxidative stress such as membrane lipid peroxidation, protein oxidation, enzyme inhibition and damage to nucleic acids (Mishra *et al.,* 2008). There is some evidence that ethylene may act as amplifier to ROS (Bouchez *et al.,* 2007; Montero-Palmero *et al.,* 2013).

Results may be supported by previous studies that reported increasing ACC contents in roots and leaves in tomato plants after 21 days on salinised medium (Dodd *et al.* 2012). Likewise, Siddikee *et al.* (2011) reported higher ACC levels in roots of red pepper plants under salt stress

for 7 days. Results were in contrast with Ben Salah *et al.* (2013) who stated a decrease in ACC content after 21 days of salt stress in roots and leaves of the salt-tolerant *Medicago ciliaris* plant. Han *et al.* (2013) found no clear correlation between Cd exposure and ACC content in leaves of the halophyte *Kosteletzkya virginica*.

3.11 The effect of heavy metals on the appearance and plant height

Tolerance to Zn and Cd at the whole plant level was examined *in vivo*. Plantlets were transferred to pots containing polluted soil at the concentrations 10 and 1.0 mg/kg soil of Zn and Cd respectively. Results showed no variation at the morphological and plant height bases between plants grown in the contaminated soil with the control after 21 days, indicating the potential of *R. graveolens* to tolerate such high concentrations of Zn and Cd. It is also concluded the possibility of this plant to grow as a ground cover on the contaminated sites in such heavy metals.

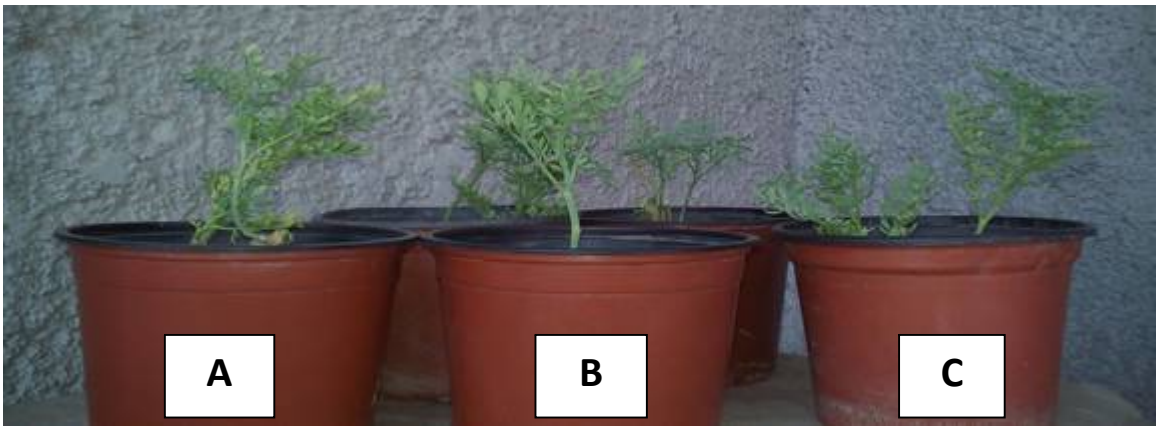

Figure 3.15 The effect of Zn and Cd on the appearance and plant height. A: control treatment. B: *R. graveolens* plantlet grown in soil contain Zn at the concentrations 10 mg/kg soil. C: *R. graveolens* plantlet grown in soil contain Cd at the concentrations 1 mg/kg soil.

Chapter Four

Conclusions

and

Recommendation

Conclusions

1. *Ruta graveolens* callus cultures can be induced on MS medium supplemented with 1.0 mg/l 2,4-D and 1.0 mg/l kin, so as with 1.25 mg/l 2,4-D and 1.0 mg/l kin.
2. Callus cultures and regenerated shoots were tolerant to heavy metals Zn, Cd, and Fe at concentration 1 ppm in the order Zn > Fe > Cd.
3. Further increasing in heavy metals concentrations affect callus cultures adversely.
4. Analysis of *Ruta graveolens* tissues revealed its ability to accumulate moderately high concentrations of heavy metals (7100 µg/g Zn, 440 µg/g Fe, and 59 µg/g Cd).
5. Microshoots regeneration achieved efficiently on half strength MS medium supplemented with 2.5 mg/l BA.
6. PCR analysis revealed the presence of ACC synthase gene (*ACS2*) which encodes to ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) synthesis.
7. Agarose gel electrophoresis documentation demonstrated a variation in bands molecular sizes between control and treatments using specific primer pairs Pp400.
8. The differences in bands molecular sizes exhibited in *Ruta graveolens* samples that were subjected *in vitro* to salt and heavy metals stresses using specific primer pairs Pp400 may belong to ROS accumulation that may be resulted in a mutation.

Recommendations

1. Analysis of ACC content in *Ruta graveolens* tissues under adverse environmental conditions.
2. Investigation of ACS2 gene expression using RT-PCR technique.
3. Investigation of the band detected in control sample when using primer pairs Pp400.
4. Investigation the type of mutation using DNA sequencer.
5. Studying the ability of *Ruta graveolens* cultures to accumulate other heavy metals such as selenium (Se), copper (Cu), and nickel (Ni)

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المصادر باللغة العربية

انعام خلف عيسى، منتهى نعمه الثويني، ثائر ناصر داود. تقرير مستوى بعض العناصر الثقيله في مياه الصرف الصحي المعالجه المعاده الى نهر ديالى من محطة الرستميه في بغداد. مجلة كلية التربيه الاساسيه. العدد الخامس و السبعون (2012).

النموات الخضرية التي إستطالت في وسط MS بنصف القوه مضافاً له 0.1 ملغم/لتر من اندول حامض البيوتريك (IBA) لتجذيرها. خضعت العينات للتحليل بوساطة تقنية PCR من أجل التحري عن الأسس الجزيئية لتحمل المعادن الثقيلة في المزارع النسيجية لنبات السذاب. أنتخب جين 1-امينوسايكلوبروبان-1- حامض الكربوكسيللك (ACC) ساينثيز والمعروف ACS2 gene للبرتقال الحلو (*Citrus sinensis*) لتصميم زوجين من البادئات لإختبار PCR وذلك من أجل الكشف عن وجود الجين في نبات السيطرة و تلك التي عُرضت للمعادن الثقيلة وتم تسمية البادئات Pp600 و Pp400. بينت نتائج تحليل PCR بان عينة السيطرة قد أعطت ناتج بحجم جزيئي ~ 600 زوج قاعدي عند إستعمال زوج البادئات Pp600 وأعطت حجم جزيئي ~ 400 زوج قاعدي بإستعمال زوج البادئات Pp400 مما يؤكد وجود جين ACS2. دُرس تأثير الشد الجفافي والملحي والمعادن الثقيلة على جين ACS2 وبينت النتائج ان جميع المعاملات اعطت ناتج بحجم جزيئي ~ 600 زوج قاعدي عند إستعمال زوج البادئات Pp600. أوضحت النتائج ظهور نواتج PCR بحجوم مختلفة عند إستعمال زوج البادئات Pp400. أعطت العينات التي عُرضت للجفاف والمعادن الثقيلة (Fe, Zn او Cd) نواتج بحجم جزيئي ~ 400 زوج قاعدي في الوقت الذي أعطت العينات التي عُرضت الى الإجهاد الملحي و Fe نواتج PCR بحجم جزيئي~ 213 زوج قاعدي. واخيرا اعطت العينات التي عرضت للملوحة و Zn نواتج PCRبحجم جزيئي ~ 226 زوج قاعدي. أجريت التجربه الحقلية للتحري عن قابلية النبات لتحمل المعادن الثقيلة داخل الجسم الحي. يستنتج من الدراسة الحالية إمكانية زيادة تحمل نبات السذاب للمعادن الثقيلة وتوظيفه في زراعة المواقع الملوثة وكذلك الأراضي المتأثرة بالملوحة والجفاف وتم تأكيد وجود جين التحمل في الزروعات المدروسة.

الخلاصة

نفذت عدة تجارب للتحري عن قابلية تحمل نبات السذاب *Ruta graveolens* لتحمل المعادن الثقيلة خارج الجسم الحي ولتقصي الأسس ألجزيئية لتحمل تلك العناصر.أستحث الكالس من أجزاء السوق المزروعة في وسط موراشج وسكوك (Murashige and Skoog, 1962) المجهز بتراكيز مختلفة من 4,2 داي كلوروفينوكسي اسيتك اسد (2,4-D) بالتراكيز0.0، و0.75، و1.0، أو 1.25 ملغم/ لتر والكاينتين (kin) بالتراكيز0.0، و 0.75، و 1.0، أو1.25ملغم/ لتر. أعطت التوليفة 1 ملغم/لتر من 2,4-D + 1 ملغم/لتر من kin و التوليفة 1.25 ملغم/لتر من 2,4-D + 1.00 ملغم/لتر من kin أعلى نسبة مئوية لتحفيز الكالس وصلت 100 و 93 % على التوالي. أُديم الكالس في وسط MS المجهز بمقدار 1.00 ملغم/لتر 2,4-D و 0.5 ملغم/لتر kin. أعيد زراعة قطع الكالس الناشئة على الأجزاء النباتية للسوق على وسط ادامة مجهز بالمانيتول بالتركيز 300 ملغم/لتر لتحفيز الإجهاد الجفافي او بماء مالح بتوصيل كهربائي مقداره 30 دسيمنز/م لتحفيز الإجهاد الملحي. أنتخب الكالس المتحمل للجفاف والملوحة و تم اخضاعه لتراكيز مختلفة(0 .0، و 0.1، و0.5، و1.0، و2.0 أو 3.0 جزء بالمليون) من الزنك، الكادميوم، أو الحديد. بينت النتائج حصول نقصان في وزن الكالس الطري مع زيادة تراكيز المعادن. حُسب مقياس التحمل بعد تعريض الكالس لتراكيز وفترات مختلفة من الإجهاد. سُجلت اعلى القيم تحت تركيز 1 جزء بالمليون من المعادن الثقيلة بعد مرور 63 يوما بعد تعريض الكالس للمعادن ما عدا في حالة الكالس المتحمل للملوحة اذ سُجلت اعلى قيمة تحمل بعد مرور21 يوما تحت إجهاد الكادميوم. أستعمل مطياف الإمتصاص الذري 5000 لتقدير تراكيز المعادن داخل عينات الأنسجة التي شملت أوراق النبات النامي في تربة اعتيادية و نوعي الكالس المعرض للإجهاد وغير المعرض للإجهاد. بينت النتائج بان المزارع النسيجية لنبات السذاب تتحمل تراكيز عالية لحد ما من المعادن الثقيلة (7100 من الزنك ، 440 من الحديد و 95 مايكروغرام/غرام من الكادميوم). أنتخب الكالس المتحمل للمعادن الثقيلة وتم إخلاف النموات الخضرية منه في وسط MS بنصف القوه والذي جُهز بالسايتوكاينين بنزل ادينين (BA) بالتراكيز0.0, و 2.5 او 3.0 ملغم/لتر ونفثالين حامض الخليك (NAA) بالتركيزين 0.0 او 0.5 ملغم/لتر. أعطى BA بالتركيز 2.5 ملغم/لتر اعلى نسبة مئوية من الافرع بعد 8 اسبوع. تحققت إستطالة النموات الخضرية وتضاعفها في نفس وسط الإخلاف المعزز بمقدار 0.5 ملغم/لتر من حامض الجبرلين (GA_3). أختبرت النباتات التي تم إخلافها عن مدى تحملها للمعادن الثقيلة بعد زراعتها في وسط MS مضافاً له 1 جزء بالمليون من المعادن الثقيلة. زُرعت

الاهداء

إلى من لا يطيب الليل إلا بشكره ... ولا يطيب النهار إلا بطاعته ... ولا تطيب الاخرة إلا بعفوه ...

ولا تطيب الجنة إلا برؤياه ... خالقي ... الله ﷻ

إلى من بلغ الرسالة ... وادى الامانة ... ونصح الامة ... إلى نبي الرحمة و نور العالمين ...

سيدنا محمد ﷺ

إلى روح من أحمل إسمه بكل فخر ... إلى من أسس لي درب النجاح و كان سندي في الحياة ...

اليك يا من أودعتني لله ... أبي رحمك الله

إلى الشمعة التي ذابت بكل كبرياء لتنير كل خطوة في دربي ... إلى رمز الحب و بلسم الشفاء ...

إلى ينبوع الصبر والتفاؤل والامل ... أمي الحبيبه

إلى القوب الطاهرة الرقيقة ... إلى رياحين حياتي ورفيقات دربي ... أخواتي (منى، ندى و نغم)

إلى سندي و قوتي و ملاذي بعد الله ... إلى من اثروني على أنفسهم ... إخوتي (عمر، عثمان،

علي وصفاء)

إلى كل من أراد لي الخير و لم تذكره أسطري

زينب

بسم الله الرحمن الرحيم

نَرۡفَعُ دَرَجَٰتٖ مَّن نَّشَآءُۗ
وَفَوۡقَ كُلِّ ذِي عِلۡمٍ عَلِيمٞ ٧٦

صدق الله العظيم

سورة يوسف

الاية 76

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة النهرين / كلية العلوم

قسم التقانة الأحيائية

التحري عن قابلية نبات السذاب *Ruta graveolens* لتحمل المعادن الثقيلة خارج وداخل الجسم الحي

رسالة

مقدمة الى كلية العلوم- جامعة النهرين وهي جزء من متطلبات نيل

درجة الماجستير في علوم التقانة الأحيائية

من قبل

زينب محمود لطيف

بكالوريوس تقانات كيميائية-أحيائية (2007)

بإشراف

أ.د. كاظم محمد ابراهيم **أ.م.د. عبد الواحد شمخي جابر**

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