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

Effect of Abiotic Stress on The Accumulation of Some Metabolites in *Ruta graveolens (In Vitro)*

A Dissertation

Submitted to the College of Science, Al-Nahrain University as a Partial Fulfillment of the Requirements for the Degree of Doctorate of Philosophy in Biotechnology

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October 2014

Dhul Hijjah 1435

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيم

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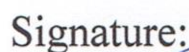
Signature: 

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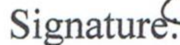
Signature: 
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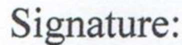
Signature: 
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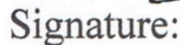
Signature: 
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Acknowledgments

I would first like to thank Almighty and Merciful "Allah" for everything. Most importantly, I thank him for support that without it I would not be where I am today.

I would like to express my deep thanks to my supervisors, Prof. Dr. Kadhim M. Ibrahim and Dr. Shatha I. Yousif, for their advice, guidance, support and tremendous help throughout my study.

I express my sincere thanks to all members of Agricultural Research Directorate, Ministry of Science & Technology, Baghdad- Iraq.

My deep appreciation to Mr. Majeed Hameed and Mohamed Jaffir in the Ministry of Science and Technology for their helps in HPLC analysis.

Finally, I express special appreciation to my Husband Ahmed for his support and encouragement. Also my thanks go to my family specially my kids Ali and Hussein who helped to raise my morale during the difficult time.

Summary

Structural and functional characterization of environmental stress-induced genes has contributed to a better understanding of how plants respond and adapt to different abiotic stresses by using DDRT-PCR, with the aim of increasing salinity and drought tolerance in *Ruta graveolens* by employing plant tissue culture technique. A significant increase in the mean response % for callus induction in leaf and stem explants of *Ruta* was achieved using Murashige and Skoog (1962) medium (MS) supplemented with different concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D) and Kinetin (Kin). The highest response for callus induction on leaf explants reached 96.3% at 1.5 mg/l 2,4-D and 0.5 mg/l Kin, while for stem explants reached 100% at 1 mg/l 2,4-D and 1.0 mg/l Kin. Callus proliferation was recorded at 1.0 mg/l 2,4-D and 0.5 mg/l Kin. The highest shoot regeneration was recorded at 1.5 mg/l BA and 0.5 mg/l NAA. Highest shoot length was achieved at 0.5 mg/l GA_3. Shoots were transferred onto a half strength MS medium supplemented with 0.1 mg/l IBA achieving 100% rooting. Many experiments were carried out for screening *R. greaveolens* calli tolerance for drought using mannitol and saline water using direct and gradual screening methods. *In vitro* callus cultures were exposed for both direct and stepwise screening to different concentrations of mannitol (0.0 – 300 g/l). Direct screening revealed a significant decrease occurred in the mean of callus fresh weight, while the highest callus fresh weight was recorded at 240 g/l mannitol reached 188 mg after 12 weeks. Gradual screening recorded maximum callus fresh weight at 300 g/l mannitol reached 281 mg after the 5th reculture. *In vitro* callus cultures were exposed to both direct and stepwise concentrations of saline water (5.0 – 30.0 $dS.m^{-1}$). Direct screening revealed a significant decrease in the mean of callus fresh weight, while a significant increase in callus fresh weight was recorded in response to 25 $dS.m^{-1}$ of saline

water reached 233 mg after twelve weeks compared with the control at 5.0 dS.m^{-1} of saline water. Gradual screening recorded maximum value of callus fresh weight at 30 dS.m^{-1} saline water reached 669 mg after the 5th reculture. The highest calli regeneration % recorded 85.03% for callus cultures exposed gradually up to 300 g/l mannitol, while reached 69.63% when exposed gradually to saline water. Porline concentrations increased in regenerated shoots derived from callus cultures subjected to both direct and gradual concentrations of mannitol and saline water. Gallic acid is markedly increased in all stressed plantlets subjected to both direct and gradual selection, but the direct exposure to saline water at 25 dS.m^{-1} recorded the highest value reached 22.6 mg/g fwt. Rutin content in shoots regenerated from direct exposure of calli to 25 dS.m^{-1} saline water recorded the highest value reached 311 mg/g fwt. Direct exposure to saline water and gradual exposure to mannitol for calli increased the concentrations of xanthotoxin in the regenerated shoots reached 12.3 and 11.3 mg/g fwt respectively. Highest concentration of bergapten (2.29) mg/g fwt was recorded in shoots regenerated from calli exposed to 240 g/l mannitol. The gain in salt tolerance in plantlets regenerated from calli treated previously with 25 dS.m^{-1} saline water directly and 30 dS.m^{-1} saline water gradually increased to 75% and 80% respectively after re-subjected to 25 and 30 dS.m^{-1} saline water for 40 days respectively. While the gain in drought tolerance in plantlets regenerated from calli treated previously with 240 g/l mannitol directly and 300 g/l mannitol gradually increased upto 70% and 72% respectively after reculture on a medium containing 240 g/l mannitol and 300 g/l mannitol for 40 days respectively. Differential display reverse transcriptase polymerase chain reaction (DDRT-PCR) was used as a powerful technique for analyzing differences in gene expression between drought and salinity stressed and unstressed (control) plantlets of *R. graveolens.* A new band with different

molecular sizes were observed in the primers OPA-01, OPA-08, OPA-11, OPA-17 and OPA- 15 indicating the expression of a new genes amplified under stress conditions or may due to already existing genes.

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List of Abbreviations

Abbreviation	Full name
2,4-D	2,4-Dichlorophenoxyacetic acid
BA	Benzyl adenine
Bp	Base paire
cDNA	Complemantry Deoxyribonucleic acid
CTAB	Cetyltrimethylammonium bromide
DDRT-PCR	Differential display reverse transcription polymerase chain reaction
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
EC	Electrical conductivity
EDTA	Ethylenediamineteraacetic acid
IBA	Indole 3-butyric acid
HPLC	High performance liquid chromatography
Kin	Kinetin
LSD	Least significant difference
mM	Milli Molar
MS	Murashige and Skoog medium
mRNA	Messenger ribonucleic acid
NAA	Naphthaleneacetic acid
n.s	Non significant
P	Propability
NaOCl	Sodium hypochlorite
PCR	Polymerase Chain Reaction
PEG	Polyethylen glycol
RAPD	Random Amplified Polymorphic DNA

RNA	Ribonucleic acid
RT	Reverse transcriptase
TAE	Tris-acetic acid EDTA
TBE	Tris-borate EDTA
TE	Tris-EDTA
T.D.S.	Total dissolved salts
T.S.S.	Total soluble salts
T.H.	Total hardness

Chapter one

Introduction

and

Literature Review

1. Introduction and literature review

1.1 Introduction

Environmental stresses, such as drought, salinity, toxic compounds, cold and heat cause adverse effects on the growth and productivity of crops. Abiotic stress is the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50% (Rodriguez *et al.,* 2005).

More than 800-1000 million hectares of land throughout the world are salt affected, this area accounts for more than 6 - 10% of the world's total land area. Most of salt affected land have arisen after the accumulation of salts over long periods of time in arid and semiarid zones (Dinar, 2009). Drought and salt stresses, together with temperature, are the major problems for agriculture because these adverse environmental factors prevent plants from realizing their full genetic potential, which greatly affect plant morpho-physiology and ultimately leads to reduction in yield (Balal *et al.,* 2011).

Salinity affected lands have increased in recent years in Iraq, out of 43.5 million ha of soils, 1.3 million ha are slightly salt effected soils and 6.7 million ha are severely salt effected soils (Hachicha and Abdelgawed, 2003). Such lands are either un-utilized or semi-utilized, because of high salinity in irrigation water and soil, lack of rain and high evaportranspiration rates causing desertification and dust storms in Iraq. In general crops are affected by soil salinity when exceeds than 4 $dS.m^{-1}$.

Massive efforts have been devoted to develop techniques for breeding crops with better performance under saline conditions (Abdi *et al.,* 2011).

Selection for salt tolerance at the cellular level has proven to be an efficient system (Darvishi and Farahani, 2010).

The genus *Ruta graveolens* belongs to the family Rutaceae, which includes important taxa used for food, flavors, fragrances, and colorants. *Ruta* is a medicinal plant whose roots and aerial parts contain more than 120 natural products of different classes such as acridone, alkaloids, coumariens, essential oil, flavonoids and furoquinoline (El-Sherbeny *et al.,* 2007).

In vitro techniques have been incorporated in breeding programs and offer considerable opportunities for plant genetic improvement saving space and time required by conventional methods, preliminary physiological information about the behavior of the plant cells under stress conditions (Bouiamrine and Diouri, 2012). Cell lines with desirable agricultural traits, such as salt and drought tolerance (Vazquez–Flota and Loyola–Vargas, 1994). Differential display reverse transcriptase polymerase chain reaction (DDRT-PCR) is a powerful technique for analyzing differences in the expression of defense- related genes involved in salinity and drought stress.

Therefore, with the aim of using *R. graveolens* as a ground cover in salt and drought affected lands exposed to erosion and could be for other environmental stresses, the objectives of this study were:

1. Utilization of *in vitro* selection methods under salinity and mannitol stresses for selecting tolerant cell lines of *Ruta graveolens.*
2. Investigating some secondary metabolites compounds in regenerated plants subjected previously to stress.
3. Evaluating the effect of salinity and mannitol on growth characteristic and gene expression by using DDRT-PCR method.

1.2 Literature Review

1.2.1 *Ruta graveolens* L. (*Ruta*)

R. graveolens L., common name is Rue, common Rue, garden Rue, fringed Rue, herb of grace, German Rue, Sadab or Ruda (Chevallier, 1996; Gunaydin and Savci, 2005). It is herbaceous perennial essential oil and medicinal plants, in the wild form it is widespread, originally native to the Mediterranean region from Canary Islands to Asia minor and the Near East. Also it is documented that Ruta is native to Southern Europe and Northern Africa (Asilbekova *et al.,* 1993; El-Sherbeny *et al.,* 2007; Ratheesh and Helen, 2007). Now It is widespread in temperate and tropical parts of the world including South Africa and North America where it was introduced from Europe, after the Spanish conquest (Barcloux, 2008; Gunaydin and Savci, 2005).

1.2.2 Plant taxonomy

Kingdom : Plantae

Subkingdom : Tracheobionta – vascular plants.

Superdivision: Spermatophyta – Seed plants.

Division: Magnoliophyta – Flowering plants.

Class: Magnoliopsida – Dicotyledons.

Subclass: Rosidae.

Order: Sapindales.

Family: Rutaceae – Rue family.

Genus: *Ruta* L. – Rue.

Species: *Ruta graveolens* L. – common Rue (USDA, GRIN Taxonomy for Plants).

1.2.3 Plant description

R. graveolens is a small evergreen sub shrub or semi woody perennial plant grows up to one meter in height. The stems become woody near the base, but remain herbaceous nearer the tips, the grey-

green leaves are oblong or spoon shaped segments. The plant forms dense branches (fig 1.1) and its flowers are characterized by a strong unpleasant smell.

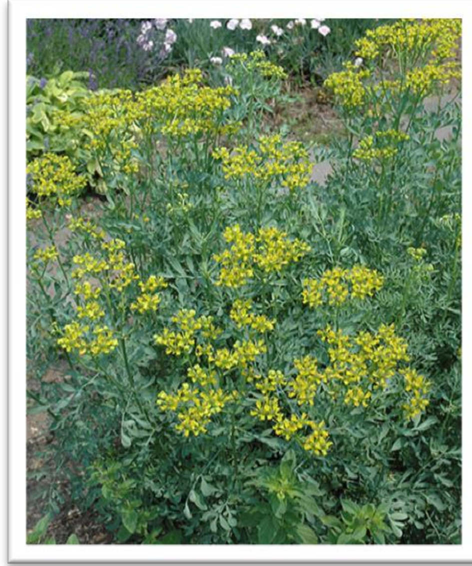


Fig 1.1 *R. graveolens* makes the plant a good candidate as a ground cover (the photo was taken by the researcher).

The aromatic smell is due to the presence of pellucid gland, the oil glands are principally present in leaves having a strong deterrent odour. The plant flowers in spring and produces small, lobulate fruits (Gunaydin and Savci, 2005; Varbanova *et al.,* 2005; Barceloux, 2008; Benazir *et al.,* 2011).

1.2.4 Plant ecology

Ruta graveolens is tolerant to a wide range of soils including soils with acidic pH (4.5-6), slightly alkaline pH (7.6-8), dry, shallow, rocky, poor sandy, sharply drained, drought and low fertility soils. *Ruta* also is adapted to sandy loam and clay loam soils. It grows well in direct sun and performs well at low levels of water, relatively tolerant to drought, pollution, seashore, slope, rabbits and deer (Http;///E:/Floridata *Ruta graveolens*.htm; Http://www.missouribotanicalgarden).

1.3 *R. graveolens* uses

Rue is an attractive herb shrub, responds well to pruning, can be shaped into a rounded mass and used in borders and beds. Rue is a culinary herbs often being used for country-style food. Although rue leaves are bitter when consumed in large quantities, small amounts of rue leaves added to cream cheese, omelets, salads and egg dishes impart a pleasant musky flavor. Also it's a very popular herb in Ethiopia, fresh rue leaves are sometimes used as a coffee flavourant, in making pickles or put in cooked dishes for a bitter taste and flavor while used in middle ages as a flavouring in food, vinegar and beverages. Rue oil has a flavor similar to the bitter oil in orange or lemon rinds which is used in cosmetics and foods (Shehadeh *et al*., 2007; Preethi *et al.,* 2008).

1.3.1 Traditional and medicinal uses

Ruta a folklore plant has been used traditionally in the middle east and south Africa for treatment of a wide variety of diseases (Varbanova *et al.,* 2005; Shehadeh *et al.,* 2007; Barcloux, 2008). Ancient Egyptians and early Greeks used rue to improve eyesight, the juice of fresh rue has been used to relieve toothaches and earaches, while in Chinese medicine rue is used to eliminate intestinal worms (Preethi *et al.,* 2008; Freire *et al.,* 2011). Rue is used as the key ingredient in homemade herbal cough syrup. Orally, rue is used as analgesic, antipyretic, antispasmodic, anthelmintic and abortifacient (Gunaydin and Savci, 2005; El-Sherbeny *et al.,* 2007; Shehadeh *et al.,* 2007; Emam *et al.,* 2009).Topically, it is used as hair tonic, insect repellent and antidote for toxins such as snake-bite, insect bite and scorpion venoms (Shehadeh *et al.,* 2007; Emam *et al.,* 2009; Freire *et al.,* 2011).

Also rue is used to treat internal infections, inflammations, eczema and external ulcers (Hale *et al.,* 2004; Shehadeh *et al.,* 2007). In Germany, extracts of rue are marketed for relief of cramps, rheumatism,

and to treat various swelling (Hale *et al.,* 2004; Ratheesh and Helen, 2007; Barcloux, 2008). Rue is used as an energizer and antibleeding to heal injuries (Hashemi *et al.,* 2011). Rue herb contains many secondary metabolites such as furocoumarins, furoquinolines and acridone alkaloids (Hale *et al.,* 2004; Gunaydin and Savci, 2005; Ahmad *et al*., 2010). Many of these compounds are physiologically active and therefore of pharmacological interest (El-Sherbeny *et al.,* 2007). The medicinal action of common rue in treatment for a sedative, anti-inflammatory, antiseptic, irritant, antifungal and stomachic (Gunaydin and Savci, 2005; El-Sherbeny *et al.,* 2007; Benazir *et al.,* 2011). The whole herb is expectorant, ophthalmic, anti-platelet, CNS-depressant and rubefacient (Gunaydin and Savci, 2005; El-Sherbeny *et al.,* 2007; Ahmad *et al*., 2010; Benazir *et al.,* 2011). The volatile rue oil possesses antibacterial activity against *Micrococcus pyogenes var. aureus* and *Escherichia coli* (Ahmad *et al*., 2010). Rue also has phytotoxic properties used to cure skin diseases like psoriasis, vitiligo, gout and leucoderma. It was officinal for hypertension, diabetes, allergic manifestations, cutaneous lymphomas and rheumatic arthritis (Bohidar *et al*., 2008; Batanouny, 2012; Diwan *et al*., 2012).

Additionally, it has wide uses in the treatment of gastric, nervous, gynecological, skin, kidney and other diseases (Asilbekova, *et al.,* 1993; Preethi *et al.,* 2008). Rue contains cardiovascular active substances that have a direct effect on the cardiovascular system (El-Sherbeny *et al.,* 2007). Recently Rue showed homeo potentiated induces cell death in brain cancer cell (El-Sherbeny *et al.,* 2000; Preethi *et al*., 2006; Preethi *et al.,* 2008).

1.4 Secondary metabolites in plants

Many higher plants produce a wide range of natural products or secondary metabolites (Karuppusamy, 2009; Mazid *et al*., 2011), which

may have important functions in the plants adaptation biotic and abiotic stresses (Terryn *et al*., 2006). Plants produce a high diversity of secondary metabolites with a prominent function in protection against predators and microbial pathogens on the basis of their toxic nature (Mazid *et al*., 2011). Many secondary metabolites are useful compounds used as pharmaceuticals, agrochemicals, flavors, in aroma industries, food additives and pesticides (Vanisree *et al*., 2004; Karuppusamy, 2009). More than 100000 secondary metabolites have been identified by restarchers belong to alkaloids, phenolics, glycosides, saponins, terpenes, peptides and lectins (Wink, 2010).

1.5 Secondary metabolites in *R. graveolens*

Rue a good source of a diverse classes of secondary metabolites. More than 120 pharmacutical active compounds related to different secondary product groups such as acridone alkaloids, coumarins, furacridone alkaloids, volatile oils, flavonoids, furocoumarins, furoquinolines are present in the plant (Oliva *et al*., 2003; Bohidar *et al*., 2008; Ahmad *et al*., 2010; Diwan and Malpathak, 2011a).

1.5.1 Phenolic compounds

Plants responds to insect feeding, pathogen infection and environmental stress with the up regulation of specific defense mechanisms. Phenolic acids and flavonoids such as salicylic acid (SA), gallic acid (GA), vanillic acid (VA) and jasmonic acid (JA) are thought to be central components of signaling pathways leading to the activation and fine tuning of these defense response (Engelberth *et al*., 2003; Tumbas *et al*., 2004). Various types of abiotic stress can enhance the production of plant secondary metabolites. Accumulation of such metabolites often occurs in plants subjected to stresses including various elicitors or signal molecules. Improvement of secondary products biosynthesis by *in vitro* cultured plant cells can be achieved by subjecting the cultured plant cells

to stress factors, especially drought stresses like PEG, sucrose, mannitol or sorbitol which have been proposed by several workers as osmotic agents for *in vitro* selection for drought tolerant plants. Salinity stress using NaCl was sleeting used for salt tolerant plants (Azhar *et al.,* 2011; Hussein and Aqlan, 2011; Ramakishna and Ravishankar, 2011; Mohamed and Ibrahim, 2012). Hussein and Aqlan, (2011) reported that low concentrations of mannitol and NaCl enhanced total phenolics, flavonoids and tannins content in *Trigonella foenum-graecum* L. callus culture compared to the control, while higher salt concentrations significantly decreased secondary metabolite accumulation. *Datura stramonium* callus showed mild increase in Scopolamine and atropine at different concentrations of NaCl (Abdel-Rahman *et al.*, 2013). Sharma and Ramawat, (2013) reported that phenolic compounds gradually elevated in NaCl-treated callus of *Salvadora persica*. Kim *et al.* (2001) found that high concentrations of mannitol, sorbitol and PEG enhanced paclitaxel accumulation in suspension cell cultures of *Taxus chinensis*.

1.5.2 Rutin

Rutin is the rhamnoglucoside or flavonol glycoside comprised of the flavonoid quercetin, and found in many medicinal plants and used for treatment of various diseases related to the vascular. Its name comes from the name of *R. graveolens*, a plant that also contains rutin, that known as antioxidant which plays a role in inhibiting some cancers and as natural compound with wide range of medicinal propertie (Sofic *et al.,* 2010; Hamad, 2012). Mohamed and Ibrahim (2012) found that higher concentration of mannitol resulted in high percentage of coumarins and rutin in rue shoot culture, but the final accumulation of these bioactive molecules decreased.

1.5.3 Furanocoumarines

Furanocoumarins or furocoumarins, are a class of organic, chemical (phenolic) and toxic compounds within the coumarins group (Maria, 2012), produced by a variety of plants. The chemical structure of furocoumarin consists of a furan ring fused with coumarin. They are coumarin derivatives, one of these classes constitute polyphenols (Karamat *et al.*, 2012). These molecules exist mainly in 4 plant families: Rutaceae, Apiaceae, Fabaceae and Moraceae (Maria, 2012; Szewczyk and Bogucka-Kocka; 2012).The most abundant linear furanocoumarins are psolaren, xanthotoxin, bergapten and isopimpinellin, whereas the angular type is mostly represented by angelicin, sphondin, and pimpinellin (Szewczyk and Bogucka-Kocka; 2012).

1.5.3.1 Biosynthesis of furanocoumarins

Despite their importance in plant life, their biosynthesis remains relatively poorly documented at the molecular level (Karamat *et al.*, 2012; Maria, 2012). Furanocoumarins play the role of phytoalexin in plants, which can be synthesized as a result of elicitation by microorganisms, insects, fungi as well as a biotic elicitors such as UV radiation, environment pollutants (Szewczyk and Bogucka-Kocka; 2012).

The biosynthesis of the furanocoumarins in plants presents a double origin: the coumarins nucleous derived from aromatic amino acids (Bohlmann *et al.*, 1994) and produced *via* the shikimic acid biosynthetic pathway beginning with the conversion of phenylalanine to trans cinnamic acid and finally to umbelliferne (Berenbaum *et al.*, 1991; Maria, 2012; Szewczyk and Bogucka-Kock, 2012) by phenylpropanoid pathway and the furanic – ring derives from mevalonate pathway (Berenbaum *et al.*, 1991; Maria, 2012). The immediate precursors for furnocoumarin synthesis are umbelliferone which is the precursor to both linear and angular furnocoumarins (fig1.2) Two categories of furnocoumarins are

produced ; the linear furano-coumarins have the furan ring in line with the benz-2-pyrone nucleus, while the angular furnocoumarins have the furan ring oriented at an angle nucleus (Berenbaum *et al*., 1991; Maria, 2012, Szewczyk and Bogucka-Kock, 2012). Psoralen is the final yield of umbelliferone pathway in linear furnocoumarins, psoralen can act as a precursor for the further substituted furnocoumarins bergapten, xanthotoxin and isopimpinellin (Maria, 2012; Szewczyk and Bogucka-Kock, 2012).

1.5.3.2 Biological activities of furnocoumarins

Due to their biological activities, furanocoumarins are very interesting compounds and widely investigated. The various biological and pharmacological activities of cumarins, have been known for a long time (Karamat *et al*., 2012, Szewczyk and Bogucka-Kock, 2012). Defensive activity of furanocoumarins consists in their toxicity against phytopathogens, linear furanocoumarins particulary psoralen and its deviatives (5-methoxy psoralen) bergapten, (8-methoxy psoralen) xanthotoxin and isopimpinellin have been effectively and currently used for the treatment of skin diseases because of its photosensitization towards UV light (Massot *et al*., 2000; Szewczyk and Bogucka-Kock, 2012), these compounds are facilitate or promoting skin pigmentation and treating psoriasis or severe blemishes (vitiligo) exist (Massot *et al*., 2000; Lievre *et al.,* 2005; Orlita *et al*., 2008; Sidwa-Gorycka *et al.,* 2009; Szewczyk and Bogucka-Kock, 2012).

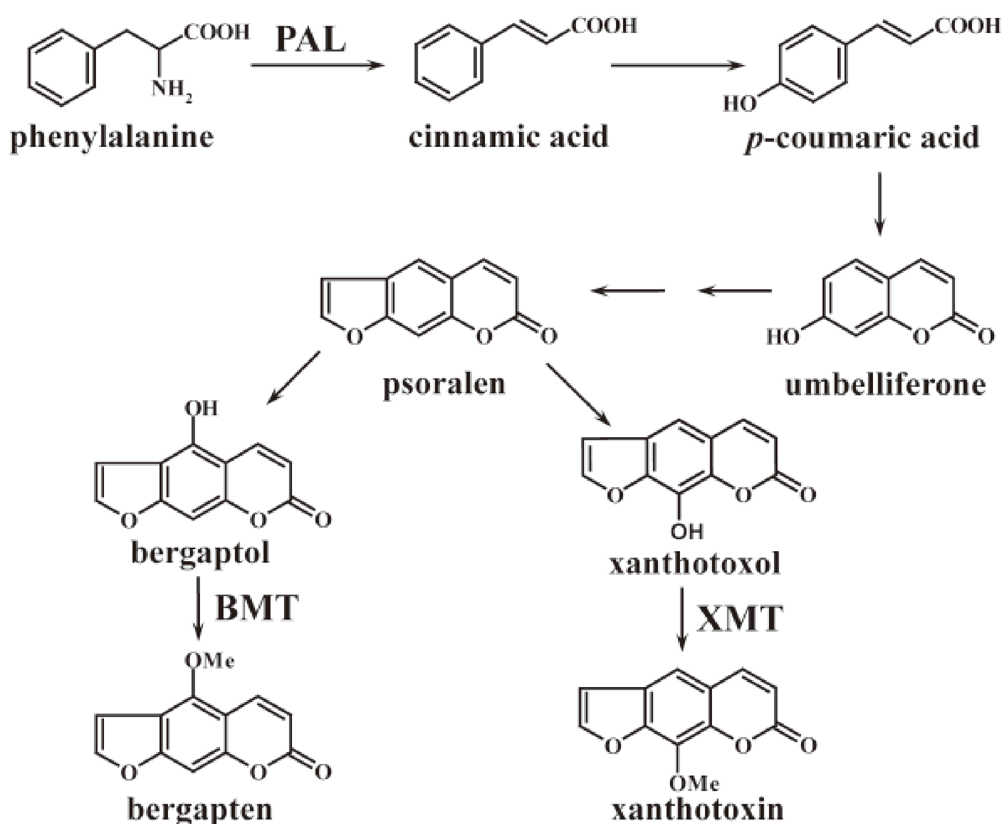


Fig 1.2 Schematic outline of linear furanocoumarine biosynthesis (Hehmann *et al*., 2004; Maria, 2012).

Although the combination of these compounds with ultraviolet light are used for antieoplastic effects and for treating skin disorders, including alopecia, cutaneos T-cell lymphoma (Diwan and Malpathak, 2008; Diwan and Malpathak, 2010; Sidwa-Gorycka *et al.,* 2009; Szewczyk and Bogucka-Kock, 2012), leucoderma (Diwan and Malpathak, 2010; Diwan and Malpathak, 2011b; Sidwa-Gorycka *et al.,* 2003), excema, lichen planus (Szewczyk and Bogucka-Kock, 2012), and also in neurology (the symptomatic treatment of demyelinatiog diseases, particulary multiple sclerosis) (Orlita *et al*., 2008; Sidwa-Gorycka *et al.,* 2003; Sidwa-Gorycka *et al.,* 2009). They have recently been demonstrated to be potent anti-HIV agent (Diwan and Malpathak, 2011b).

The research showed that homeopathically potentiated *Ruta* induces cell death in brain cancer cells (Preethi *et al*., 2006; Szewczyk and

Bogucka-Kock, 2012). Xanthotoxin and bergapten are active against fungal plant pathogens (*Rhizoctonia solanii*, *Fusarium* spp., *Penicillium* spp., *Trichoderma viride*) (Oliva *et al*., 2003), because of its medicinal properties furanocoumarins have gained wide applications in pharmaceutical industry (Hehmann *et al.*, 2004; Diwan and Malpathak, 2008).

1.6 Stresses

Stress is defined as an external factor that exerts a disadvantageous influence on the plant, or any changes under or above optimum growth conditions which disrupt the normal physiological function or metabolic homeostasis. The need for adjustment of metabolic pathways in a process usually referred to as acclimation (Bressan, *et al*., 2002; Munns, and Tester, 2008; Vázquez and Linacero, 2010). Increased sunlight leads to the generation of reactive oxygen species, which damage the plant cells, the threat of global environment change makes it increasingly demanding to generate crop plants that could withstand such harsh conditions (Xiong and Ishitani, 2006). Stress may result from changes in abiotic factors (fig1.3), such as climatic factors, mechanical damage and chemical or physical modifications in the environment and biotic factors, all these factors be able to alter plant metabolism leading to negative effects on growth development and productivity of plant. Plants cannot escape abiotic stresses, enable plants to develop unique molecular mechanisms to cope with such stresses they alter their physiologies, metabolic mechanisms, gene expression and developmental activities to tolerate abiotic stress (Madhavarao *et al.,* 2006; Vázquez and Linacero, 2010).

1.6.1 Similarity between salinity and drought stresses

Salinity and drought stresses show a high degree of similarity with respect to physiological, biochemical, molecular and genetic effects (Leksungnoen, 2012). Both stresses lead to cellular dehydration, which

causes osmotic stress and removal of water from the cytoplasm into the intracellular space resulting in a reduction of the cytosolic and vacuolar volumes. Early responses to water and salt stress are largely identical except for the ionic component in the plant cells under salt stress that causes ion imbalances (Razmjoo *et al.*, 2008; Leksungnoen, 2012; Muszynska *et al.*, 2014). These similarities include metabolic processes, e.g., a decrease in photosynthesis or increase in levels of the plant hormonal processes such as abscisic acid (ABA). High intracellular concentrations of sodium and chloride ions are an additional problem of salinity stress (Balal *et al.*, 2011; Leksungnoen, 2012). Thus, plants may use common pathways and components in the stress response relationship known as cross-tolerance, which allows plants to acclimate to a range of different stresses after exposure to one specific stress.

The common signals and elements are found as plants are exposed to salinity, cold, or drought tresses (Leksungnoen, 2012), the sensing of these stresses induces signaling events that activate ion channels, kinase cascades, production of reactive oxygen species, and accumulation of hormones (fig 1.3) (Perez- Clemente and Gomez – Cadenas, 2012; Wani *et al.*, 2013). Thus, a salinity tolerant species could also be drought tolerant or vice versa (Leksungnoen, 2012). These signals ultimately induce expression of specific genes that lead to the assembly of the overall defense reaction (Dogan *et al.*, 2010; Perez- Clemente and Gomez – Cadenas, 2012).

1.6.2 Mechanisms of plant tolerance to abiotic stresses

Salinity, drought and temperature are the major problems for agriculture (Jakab *et al.*, 2005), these abiotic stresss lead to a series of morphological, physiological, biochemical and molecular mechanisms that limiting plant growth and productivity (fig 1.3) (Rodriguez *et al.*, 2005; De Oliveira *et al.,* 2011; Perez- Clemente and Gomez – Cadenas,

2012). Xiong and Ishitani, 2006 and Peleg *et al*. 2011 reported that tolerance relies on the inherent ability of the plant to sustain growth even when conditions are unfavorable for the maintenance of basic plant processes levels. The degree of tolerance various from plant to another but these tolerance mechanisms start with stress perception followed by formation of gene products that are involved in cellular protect and repair (Bressan, *et al*., 2002; Madhava Rao, 2006).

Cramer *et al.* 2011 and Peleg *et al*. 2011 showed that the response to stress depends on the duration and severity of the event (acute vis chronic) can have a significant effect on the complexity of the response, as well as the age and development stage of the plant which varies with species and genotype level.

There are three levels of response: (1) The immediate stress reaction, which represent the first line of defense, mediated by the cellular defense mechanisms that are evolutionarily well conserved by prevented or alleviated damage in the cell. (2) Adjustments to reach a new equilibrium that including sensing and signaling circuits, initiates the immediate response, establishes a new equilibrium for further growth by homeostatic conditions for the new stressful environment, and prepares for long-term changes. (3) Responding to stress signaling and the concomitantly altered hormonal state are developed changes that may provide the best answers to long - term stress, the growth of the cell must be resume albeit at a reduced rate (Bohnert and Cushman, 2002; Rodriguez *et al.,* 2005; Lokhande and Suprasanna, 2012).

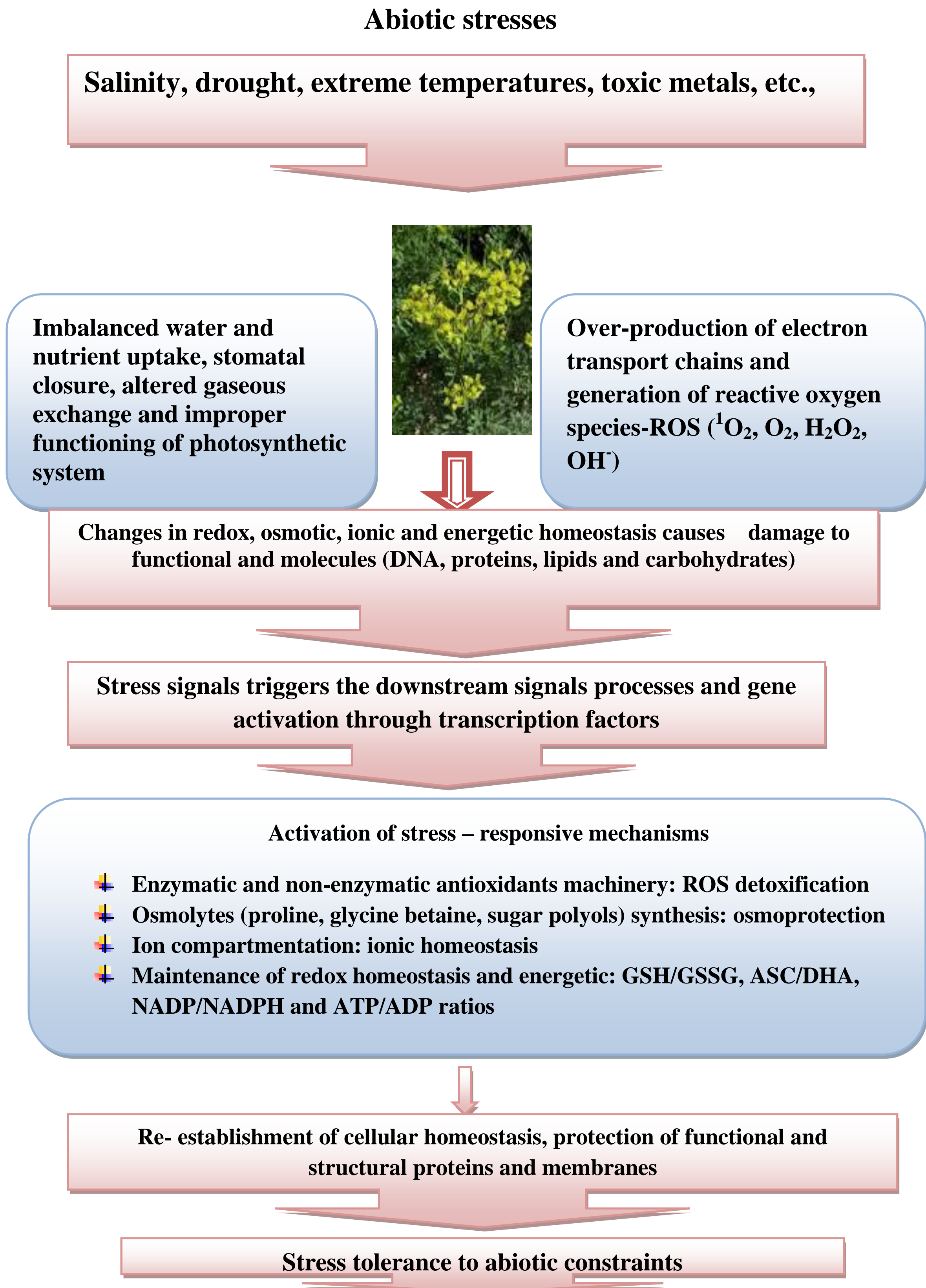


Fig 1.3 Generalized scheme for plant responses to abiotic stresses and mechanism of stress tolerance (Lokhande and Suprasanna, 2012).

The main biochemical strategies are induction of antioxidative enzymes, synthesis of compatible organic solutes or scavenging of radical oxygen species, ion homeostasis and controlled of ion uptake. Salt, drought, heat and cold stress are accompanied by the formation of reactive oxygen species ROS such as super oxide, hydrogen peroxide and hydroxyl radicals, causing extensive cellular damage and inhibition of photosynthesis. In order to cope with continuous ROS production plants have a battery of enzymatic and nonenzymatic antioxidants, which function as an extremely efficient cooperative system.

The major scavenging mechanisms include enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductas (GR), while the levels of nonenzymatic radical scavengers such as ascorbate, glutathione, flavonoids, carotenoids, anthocyanins, sugar polyols increase and can complement the existing enzyme–based protection systems. The solutes include simple or complex sugars, sugar alcohol, polyols, inositols, sucrose, glycine betaine and proline serve as osmoprotectants under stress conditions (Bohnert and Cushman, 2002; Rodriguez *et al*., 2005; Carvalho, 2008; Abdul-Jaleel, 2009; Summart *et al.,* 2010; Rehem *et al*., 2012; Wani *et al*., 2013; Jogaiah *et al*., 2014; Khan *et al*., 2014).

Rodriguez *et al*. 2005 and Fujii *et al.* 2007 reported that during abiotic stress, plants produce increased amounts of hormones such as ABA, this phytohormone is a key factor in regulating developmental and physiological processes in plants as well as in controlling many abiotic stress responses reported by Bressan, *et al*., 2002, while Rehem *et al.* (2012) stated that ABA is considered a "stress hormone" because plants respond to environmental challenges such as water and salt stress with changes in the availability of ABA.

There are many proteins that may protect macromolecules and membranes in plant cell, late embryogenesis abundant LEA proteins accumulate under conditions of extreme desiccation in higher plants water deficit, high osmolarity, and low temperature stress results in the accumulation of a group of LEA proteins (Torres *et al*., 2007; Amudha and Balasubramani, 2011). Responses to abiotic stresses require the production of important functional proteins, such as those involved in the synthesis of osmoprotectants, and regulatory proteins operating in the signal transduction pathways, such as kinases and transcription factors (TFs), because most of these responses imply the control of gene expression (De Oliveria *et al.,* 2011), Chaved and Oliveria 2004 showed that TFs play a critical role in the abiotic stress responses. Some of these TFs are controlled by ABA, while others are not, indicating the involvement of both ABA-dependent and ABA-independent regulatory systems controlling stress-inducible gene expression (Rodrigues *et al*., 2005; De Oliveria *et al.,* 2011).

1.6.3 Proline accumulation in plant cell

Proline accumulation is a widespread phenomenon in higher plants in response to various environmental stresses and was demonstrated to be protective for plants under adverse conditions. Proline accumulation was proposed to act as a compatible osmolyte, free radical scavenger, cell redox balancer, cytosolic pH buffer, and stabilizer for subcellular structures (membranes and proteins), during various stresses, especially osmotic and salt stresses (Lv *et al.,* 2011; Rai *et al.,* 2011).

Chatzissavvidis *et al.* (2014) found a significant increase in proline concentration in *Poncirus trifoliata* explants cultured on MS medium supplemented with 12 or 16mg/l NaCl they concluded that, proline has a clear role as an osmoticum, in particular, because of its zwitterionic, high hydrophilic characteristics, proline acts as a "compatible solute" that can

accumulate to high concentrations in the cell cytoplasm without interfering with cellular structure or metabolism. Mahmoud and Ibrahim, (2012) reported a significant increase in proline concentration in *Ruta graveolens* shoot cultures grown in MS medium supplemented with 3% mannitol. Under salt stress, several functions are proposed for the accumulation of proline in tissues which include osmotic adjustment, proline serves as a storage sink for carbon and nitrogen, they reserve for growth after stress resistance, detoxification of excess mitochondrial functions, and scavenging of free radicals. Hence these organic osmolytes are known as osmoprotectants Consumption, proline biosynthesis is derived from two different precursors, Glutamine and Ornethine (Chinnusamy *et al.,* 2005).

1.7 Plant tissue culture for *R. graveolens*

1.7.1 *In vitro* callus induction

Callus cultures consist of an amorphous mass of loosely arranged thin-walled parenchyma cells arising from the proliferating cells of the parent tissue. Frequently as a result of wounding, a callus is formed at the cut end of a stem or root (Dodds and Roberts, 1985). Different media were screened to determine the most effective combination of plant growth regulators for callus induction and maintenance. Baumert *et al.* (1992) established callus culture from hypocotyle of rue on solid MS medium containing 1mg/l (2,4-D, Kin) and 3% sucrose.

Shabana *et al.* (2001) studied the effect of plant growth regulators and explants type on growth of rue callus culture and, stem explants using MS medium supplemented with 2,4-D at 1.0 mg/l; (2,4-D, Kin) at 1.0 mg/l; shoot tips on MS medium supplemented with 1.0 mg/l (2,4-D, Kin) and 0.5 mg/l (2,4-D, Kin), root cultures on MS medium using combinations of 1.0 mg/l (2,4-D, Kin) and 1.5 mg/l (2,4-D, Kin) those initiated callus, while leaf explants cultured on MS medium

supplemented with 0.5 mg/l (2,4-D, Kin) produced a significant increase in callus fresh weight. Massot *et al.* (2000) reported that calli of *R. graveolens* were initiated from leaf explants on semi solid Gambourg´s medium (B5) supplemented with 3% sucrose and 1.9 mg/l 2,4-D. While Ahmed *et al.* (2010) induced calli from rue leaf explants on MS medium supplemented with 2.2 mg/l 2,4-D. Diwan and Malpathak (2010) obtained friable and fast growing callus cultures initiated from leaf explants of rue on MS medium supplemented with a combination of (2,4-D, NAA) and (NAA, IAA), this callus was subcultured after 3 weeks on a medium supplemented with the same combination of plant growth regulators. Gurudeeban *et al.* (2011) revealed that yellowish green compact calli of rue were obtained from leaf explants on MS medium supplemented with 1.0 mg/l 2,4-D and 0.5 mg/l IAA, while Zuraida *et al.* (2014) reported that culture medium containing 0.2 mg/L NAA followed by a medium containing 0.2 mg/L 2,4-D is optimal for callus induction of *R. graveolens*.

1.7.2 *In vitro* shoot and root formation

The effect of auxin and cytokinin combination was evaluated for multiple shoot induction from callus and nodal segments of rue, BA with NAA was found effective combination for multiple shoot regeneration. Fasial *et al.* (2005) reported high shoot regeneration frequency (98.5%) on MS medium supplemented with 2.25 mg/l BA and 0.46 mg/l NAA, shoots were transferred to a half strength MS medium supplemented with 0.5 mg/l IBA for root initiation. Bohidar *et al.* (2008) reported high regeneration frequency in nodal explants cultured on MS medium enriched with a combination of 1.0 mg/l BA and 0.25 mg/l IAA, while half strength MS medium augmented with different concentrations of IAA, IBA and NAA was suitable for root initiation.

Ahmed *et al.* (2010) obtained high response of callus for inducing multiple shoots (92.3%) on MS medium supplemented with 1.68 mg/l BA and 0.186 mg/l NAA, they also found that addition of 0.1 mg/l IBA to a half strength MS medium induced roots after four weeks. Diwan and Malpathak (2011b) showed that shoot cultures established multiple shoots on MS medium supplemented with 1mg/l BA and 3mg/l IAA.

1.7.3 *In vitro* selection abiotic stress tolerance

Abiotic stresses impose a major threat to agriculture (Patade *et al.*, 2005). Therefore, the efforts to develop stress tolerant plants are of immense importance to increase crop productivity (Rai *et al.*, 2011). Recently, Plant tissue culture has the potential for selection of stress-tolerant variants using a low cost laboratory set up and also allowed a deeper understanding of the physiology and biochemistry in plants culture, fast responses and short generation time (Rai *et al.*, 2011; Perez-Clemente and Gomez – Cadenas , 2012).

In vitro culture of plant cells, clumps of callus, somatic embryos, shoot cultures, organs on a medium containing selective agents offers the opportunity to select and regenerate plants with desirable characteristics. The technique has also been effectively utilized to induce tolerance including the use of some selective agents that permit the preferential survival and growth of desired phenotypes (Rai *et al.,* 2011). Imitated calli or cells are exposed to a broad range of selective agents includes NaCl to stimulate salt-tolerant cell lines, PEG, mannitol, sorbitol and sucrose generally used as osmotic stress agents in *in vitro* cultures to stimulate drought stress (Perez- Clemente and Gomez – Cadenas , 2012; Sen, 2012). Only explants or cells capable of sustaining such environments survive in the long run and are selected. Two types of the selection methods have been suggested: (a) stepwise long-term treatment, in which cultures are exposed to stress with gradual increase in

concentrations of selecting agent and (b) shock treatment, in which cultures are directly subjected to a shock of high concentration and only those which would tolerate that level will survive (Purohit *et al*., 1998; Abdel-Rheem *et al.,* 2007; Darvishi and Farahani, 2010; Rai *et al.,* 2011). Koc *et al.* (2009) reported a significant reduction in cell growth of salt tolerant cell lines of *Citrus aurantium* when cultured on Murashige and Skoog medium containing different concentrations (6, 12 and 16 mg/l) of NaCl, while Chatzissavvidis *et al.* (2014) found a significant impacts on the growth and chemical status of *Poncirus trifoliata* apical shoot tips exposed to 0,3, 6, 9, 12 and 16g/l NaCl using a step wise selection method. EL-Yacoubi *et al.* (2010) reported that increasing NaCl concentration (0, 2, 4, 6 and 8 g/l), a gradual decrease in *Troyer citrange* callus growth and dehydration were observed. Significant effect on the survival and growth dynamics of rough lemon seedlings with gradual increase in NaCl concentration was observed by Sharma *et al.* (2013), there was a significant decrease in seed germination, seedling height, internodal length, and subsequently plant weight except the length of primary roots increased proportionally with increasing concentration of NaCl but up to a limit. In majority of salinity studies, the salt used is NaCl, however many researchers, have compared the response of other Cl^- and SO_2^- salts including KCl, Na_2SO4, and $MgSO_4$ during *in vitro* screening, hence Chen *et al.* (1980) found a different responses in *Nicotiana tabacum* callus culture when growth on seawater, synthetic saline water, NaCl and other Cl^- and SO_2^- salts.

Mannitol, sugar alcohol, non-metabolic sugar, straight chain metabolite may be considered a source of carbon, is often used to induce water-deficit conditions and leads to a cellular dehydration. It causes osmotic stress and removal of water from the cytoplasm toward vacuoles, when supplemented to *in vitro* growth medium (Kim *et al.,* 2001; Hussein

and Aqlan, 2011; Ramakishna and Ravishankar, 2011; Mohmoud and Ibrahim, 2012). Mohmoud and Ibrahim, (2012) reported a significant increase in growth index and fresh weight at 1% mannitol then decreased with increasing mannitol concentration in rue shoot culture. AL-Taha, (2013) showed a progressive reduction in callus fresh weight of sour orange in either (shock) or gradual treatments by increasing PEG at 0, 2, 4, 6, or 8 g/L concentration. Bauiamrine and Diouri (2012) demonstrated that gradual increased of PEG in medium caused a decrease of callus growth and morphogenic capacity. AL-Houssine and Diouri, (2012) reported a gradual decrease in growth and water content of durum wheat calluses, with a significant reduction in the percentage of regeneration and the number of plantlets by increased PEG concentration. Abdel-Raheem *et al.* (2007) found that regeneration capability of tomato calli affected strongly by the increasing of mannitol in the medium. Interaction treatments between mannitol and sodium chloride exhibited various effects on Fenugreek calli growth (Hussein and Aqlan 2011). Wani *et al.* (2010) reported that the capacity for callus induction and regeneration decreased under different concentration of PEG.

1.8 Differential display reverse transcriptase polymerase chain reaction (DDRT-PCR)

DDRT, is one of several methods designed for analyzing differences in gene expression, and has been used successfully in many studies to identify a new genes in various tissues or cells (Alves *et al.*, 1998; Rodríguez *et al.,* 2005). DDRT, originally developed by Linang and Pardee (1992) as the most widely employed techniques allows the identification of differentially expressed genes by comparative analysis between two populations of RNA transcripts in different tissues or cell types or in different satiations that received individual environmental influences (De Almeida *et al.,* 2012; Tripathi *et al.,* 2013).

The technique is simple, quick, sensitive and powerful tool for screening cDNA (Alves *et al.*, 1998; Rodriguez *et al.,* 2005). DDRT-PCR can also be considered useful as an efficient method for cloning and construction of cDNA libraries (De Almeida *et al.,* 2012).

1.8.1 The essential steps of Differential display reverse transcriptase polymerase chain reaction (DDRT-PCR)

Reverse transcriptase of mRNAs from cells are converted to first strand cDNAs using oligo-dT primers. The use of these primers enables the homogeneous initiation of cDNA synthesis at the beginning of the poly (A) tail for any given mRNA. Polymerase chain reaction (PCR), cDNAs are further amplified by PCR in the presence of a set of second primers that are short and arbitrary in sequence. Polyacrylamide or agarose gel electrophoresis to resolve and visualize the amplified cDNA fragments (AL-Kazaz, 2001; Liang, 2002). Peisheng *et al*. (2004) used DDRT-PCR technique to study gene expression of cotton under drought and heat stress.

Chapter Two

Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 Equipments

The following equipments were used throughout this experimental work:

Equipment	Company and origin
Autoclave	Karl, Germany
Balance	Scaltec, Switzerland
Centrifuge	Hettch, USA
Distillator	GFL, Germany
EC 214 conductivity meter	Hanna, Romania
Gel documentation	Heidolph, Germany
Gel electrophoresis system	Bio com, UK
Hot plate with magnetic stirrer	Gallenkamp, England
HPLC	Shimadzue, Japan
Laminar air flow cabinet	Iraq
Millipore filter	Millipore and Whatman, England
Micropipette	Brand, Germany
NanoDrop	Thermo, USA
pH meter	Toledo, Swezerland
Refrigerator	General, Mexico
Spectrophotometer	Shimadzue, Japan
Thermocycler	Bioneer, Korea
Ultra low freezer	GFL, Germany
Vortex mixer	Heidolph, Germany
Water bath	GFL, Germany

2.1.2 Chemicals

The following chemicals were used during the experimental work:

Chemical	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, Toluene, Glacial acetic acid, Phosphoric acid, Sulfosalicylic acid, Gallic acid and Ninhydrin reagent, CTAB, β-mercaptoethanol EDTA, Tris base, Sodium acetate, Ammonium acetate, Methanol, Isopropanol and Chloroform / Isoamylalcohol.	BDH, England
L-proline, Ethanol absolute 99.5%, NaOH and HCl.	Euroclone, Italy
Bergapten, Rutin, Xanthotoxin.	Sigma,Germany
Top DNA polymerase, dNTP Mix (dATP, dCTP, dGTP, dTTP) 10mM each, PCR Buffer, Primers (operon model). DNA ladder, Oligo$(dt)_{15}$ Primer, DEPC(0.1%). Ethidium bromide, loading buffer, Agarose 10X TBE, 10X TAE.	Bioneer, Korea Promega, USA Vivantis, Germany

2.1.3 Kits

The following kits were used during the experimental work:

Kits	Company and origin
Total RNA Mini Kit	Geneaid, USA
RT-PCR Pre Mix Kit	Bioneer, Korea

2.2 Methods

This work was carried out in the plant tissue culture Lab., Biotech. Dept., Al- Nahrain University and in the Genetic Engineering Dept., Agricultural Research Directorate, Ministry of Science and Technology, Baghdad, Iraq, during the period 1/2/2012 - 1/2/2014.

2.2.1 Studied phenotype

Ruta graveolens plants less than a year old were obtained from local nursery in Baghdad, and classified by the herbarium of the Biology Department, College of Science, University of Baghdad.

2.2.2 Medium preparation

Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) components were prepared (table 2.1) and supplemented with 30 g/l sucrose and growth regulators at different concentrations. The pH of the medium was adjusted to 5.8 using 0.1 N NaOH or 0.1 N HCl, then 8 g/l agar was added to the medium. The medium was dispensed into Petri dishes (15-20 ml/dish) after autoclaving at 121 ˚C and 1.4 Kg/cm^2 for 15 min.

2.2.3 Explants surface sterilization

Leaves and stems of *R. graveolens* plant were first kept under running tap water for 30 min and then subjected to surface sterilization using 0.0,

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. $2H_2O$	$CaCl_2.2H_2O$	440
Magnesium sulphate. $7H_2O$	$MgSO_4.7H_2O$	370
Potassium phosphate monobasic	$KH_2.PO_4$	170
Micronutrients		
Boric acid	H_3BO_3	6.20
Potassium iodide	KI	0.83
Manganese sulphate. $4H_2O$	$MnSO_4.4H_2O$	22.30
Zinc sulphate. $7H_2O$	$ZnSO_4.7H_2O$	8.60
Molybdic acid (sodiumsalt).$2H_2O$	$Na_2MoO_4.2H_2O$	0.25
Cupric sulphate. $5H_2O$	$CuSO_4.5H_2O$	0.025
Cobalt chloride. $6H_2O$	$CoCl_2.6H_2O$	0.025
Chelated Iron		
Sodium ethylene diamine tetraacetate	Na_2-EDTA	33.6
Ferrous sulfate. $7H_2O$	$FeSO_4.7H_2O$	27.8
Vitamins		
Myo-Inositol	$C_6H_{12}O_6$	100
Thiamine. HCl	$C_{12}H_{17}CIN_4OS.HCl$	0.1
Nicotinic acid (free acid)	$C_8H_{11}NO_3.HCl$	0.5
Pyrodoxine. HCl	$C_6H_5NO_2$	0.5

1.0, 1.5 or 2.0% (v/v) NaOCl (Clorox) for 5, 10, 15 or 20 min with vigorous shaking. The leaf and stem segments (1.0 cm in length) were excised aseptically and transferred to sterile agar- solidified MS medium into Petri dishes (20 ml/dish) using forceps. All steps of sterilization were

carried out under aseptic conditions using laminar air flow cabinet (Ahmad *et al.,* 2010).

2.2.4 Callus induction medium

The explants (leaves and stems) of *R. graveolens* were inoculated on MS medium supplemented with different concentrations of 2,4-D (0.0, 0.5, 1.0, 1.5 or 2.0 mg/l) and Kin (0.0, 0.5, 1.0, 1.5 or 2.0 mg/l). All cultures were incubated at 25 ± 2 ºC, 16/8 hrs (light/dark) photoperiod at a light intensity of 1000 lux. After four weeks of incubation, percentage of explants producing callus were calculated according to the following equation (Tariq *et al.,* 2008):

Percentage of callus induction on explants =

(No. of explants producing callus/ No. of cultured explants) x 100

2.2.5 Callus maintenance medium

Small pieces of the light- yellow healthy calli weighting 50 mg were subcultured onto MS medium supplemented with 2,4-D (0.0, 0.5, 1.0, 1.5 or 2.0 mg/l) and Kin (0.0, 0.5, 1.0, 1.5 or 2.0 mg/l). Callus tissues were incubated as in 2.2.4.

2.2.6 Measurement of callus fresh weight

Callus fresh weight was measured after four weeks of subculture into a callus maintenance medium under aseptic conditions.

2.2.7 Shoot regeneration medium

Callus pieces (150 mg) were transferred into the regeneration medium under aseptic conditions. The regeneration was consisted of MS medium supplemented with different concentrations of BA (0.0, 1.5, 2.0, 2.5 or 3.0 mg/l) and NAA (0.3, 0,4 or 0.5 mg/l). All cultures were maintained at

25 ± 2 ºC for 16/8hrs (light/dark) photoperiod at a light intensity of 1000 lux.

The percentage of regenerated plants was calculated according to the following equation (Tariq *et al.,* 2008):

Percentage of shoot regeneration =

$$\frac{\text{No. of calli regenerated plantlets}}{\text{No. of calli pieces cultured for regeneration}} \times 100$$

(Faisal *et al.,* 2005; Ahmad *et al*., 2010)

The number of shoots was recorded after three months of incubation for 10 replicates per treatment.

2.2.8 Shoots elongation medium

All multiple shoots obtained were transferred to shoot elongation medium consisted of MS basic salts supplemented with different concentrations of GA_3 (0.0, 0.1, 0.5 or 1.0 mg/l). All cultures were maintained at 25 ± 2 ºC for 16/8 hrs (light/dark) photoperiod at a light intensity of 1000 lux. The number of shoots was recorded after three months of incubation for 10 replicates per treatment (Gurudeeban *et al.,* 2011).

2.2.9 Rooting medium

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

2.2.10 Screening of callus cultures using MS medium supplemented with abiotic stresses

Two types of selection methods were implemented by adding mannitol or saline water (drainage water) for drought and salinity respectively (Rai *et al*., 2011).

2.2.10.1 Direct screening method for callus cultures using MS medium supplemented with abiotic stresses

Callus pieces initiated on stem explants weighting 100 mg/tube were subcultured directly into MS maintenance medium containing 1.0 mg/l 2,4-D; 0.5 mg/l Kin and supplemented with different concentrations (0.0, 6, 12, 18, 24 or 30%) of mannitol or 6, 10, 15, 20, 25 or 30 $dS.m^{-1}$ of saline water (drainage water) for drought and salinity stresses respectively. The EC of MS medium was measured by using EC conductivity meter, control treatment (without adding drainage water) the EC was 5 $dS.m^{-1}$. After four weeks, the callus fresh weight / replicate was recorded, and the selected concentration of the stress agent was determined, callus cultures were re-cultured three times on the same medium. The callus fresh weight was measured after four weeks. All cultures were incubated as mentioned in 2.2.4.

2.2.10.2 Stepwise screening method of callus on MS medium supplemented with abiotic stresses

Callus pieces produced on stem explants weighting approximately 100 mg/tube were re-cultured onto MS maintenance medium containing 1.0 mg/l 2,4-D; 0.5 mg/l Kin and supplemented with gradual increments of stress agents including 0.0, 6, 12, 18, 24 or 30% mannitol or 6, 10, 15, 20, 25 or 30 dS/m^{-1} of saline water for drought and salinity respectively. The callus fresh weight / replicate was recorded after four weeks for each concentration before reculture. All cultures were incubated as mentioned in 2.2.4 (Abdel-Raheem *et al.*, 2007; Rai *et al.*, 2011).

2.2.11 Determination of proline in *Ruta* plantlets

Proline content was determined according to Bates *et al.* 1973 in

regenerated plantlets. Leaf samples weighting 0.1 g were ground, mixed thoroughly with 3% sulphosalicylic acid (10 ml) and clarified by centrifugation, 2 ml of the supernatant was mixed with an equal volume of acid-ninhydrin and acetic acid, the mixture was incubated at 100 °C for 1 h, and the reaction was terminated in an ice bath.

The reaction mixture was extracted with 4 ml toluene and the absorbance was read at 520 nm, using toluene as a blank, proline concentration was determined from a standard curve and calculated as µM/g fresh weight. Samples were measured in triplicate.

2.2.12 Preparation of proline standard curve

Proline standard curve was plotted by using different concentrations of proline 50, 100, 150, 200, 250 and 300 µg/ml. Aliquot of 2 ml glacial acetic acid and ninhydrin reagent were added to each proline concentration, incubated at 100 °C for 30 min. The samples were rigorously mixed with 4 ml toluene, light absorption of toluene phase was estimated at 520 nm using spectrophotometer (fig. 2.1).

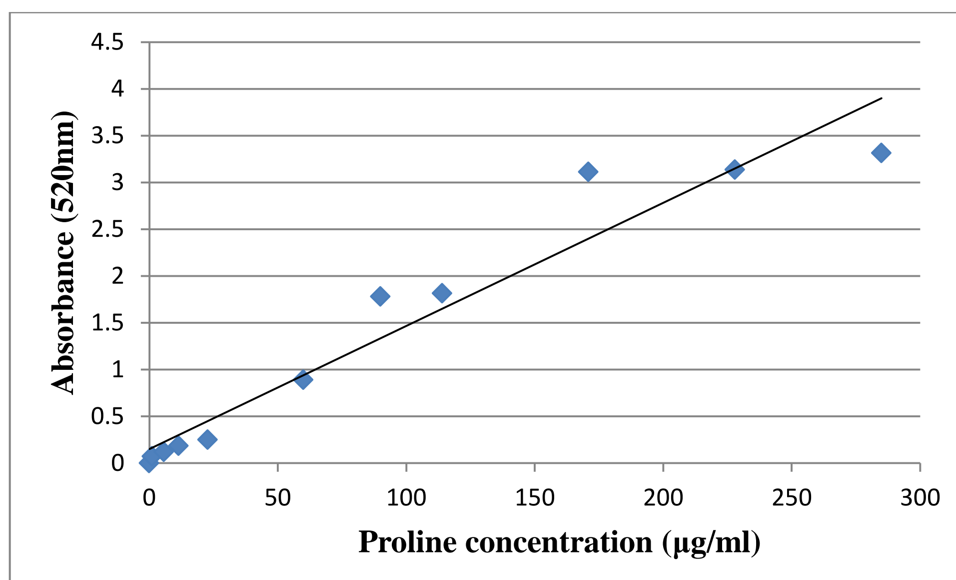


Figure 2.1: Standard curve of proline.

The proline concentration was determined from the standard curve and calculated on a fresh weight basis as follows:

[(μg proline/ml × ml toluene) / 115.5 μg /μmole]/[(g sample)/5] = μmoles proline/g of fresh weight material (Bates *et al.,* 1973).

2.2.13 Extraction of total phenolic compounds

A quantity weighting 100 mg/fresh weight leaves dissected from the plantlets (tolerant and control) was soaked into liquid nitrogen then homogenized well with 6-10 ml of the extraction solvent ethanol 50% with the aid of porcelain mortar. The extract was suspended for 30 min, then the homogenized mixture was centrifuged at 12000 rpm for 10 min, the supernatant was filtered through 0.45 μm Millipore filter unit and used for HPLC (Milesi *et al.,* 2001; Szewczyk and Bogucka-Kocka, 2012).

2.2.14 Estimation of extracted compounds

Phenolic compounds (rutin and gallic acid) and furanocoumarins (bergapten and xanthotoxin) were estimated using HPLC. They were detected by a mobile phase, methanol: water 50: 50 with a flow rate of 0.5 ml/min, the column was C18 and in wave length 300 nm (Milesi *et al.,* 2001; Mohamed and Ibrahim, 2012).

The concentrations of the investigated compounds were calculated as follows:

Peak area of extracts / Peak area of standard X standard solution concentration X total volume of extract.

2.2.15. Salinity and drought gain in plantlets regenerated from stressed callus

Plantlets regenerated from callus cultures exposed to salinity and

drought stresses and those non-treated plantlets were cultured on MS medium supplemented with saline water at 25 or 30 dS.m^{-1} respectively or mannitol at 240 or 300 g/l respectively. The survival percentage in gain experiments was calculated according to the equation:

$$\% \text{ Survival} = \frac{\text{No. of Survival Plantlets}}{\text{Total No. of Plantlets}} \times 100$$

(Oraibi, 2013).

2.2.16 Isolation of plant DNA

2.2.16.1 Reagents, chemicals and apparatus

1. CTAB extraction buffer (2% CTAB, 0.7M NaCl, 0.1M Tris-HCl pH 8, 20 mM EDTA and 1% β- mercaptoethanol, β-mercaptoethanol was added just prior to use)
2. Chloroform: isoamylalcohol solution (24:1).
3. TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0
4. Isopropanol.

2.2.16.2 DNA isolation protocol (Borges *et al*., 2009).

Genomic DNA was isolated using the following protocol:

1. Approximately 50 mg sample of dried leaves was ground with a morter and pestle.
2. The homogenized tissues were transferred to 600 µl of 2% CTAB in 1.5 ml Eppendorf tubes.
3. Incubation at 65 ºC for 30 min in a water bath.
4. Three microliters of RNase was added and incubated at 37 ºC for 60 min.
5. Aliquot 200 µl chloroform: isoamyl alcohol (24:1) was added to the solution, and mixed well. The emulsified mixture was

centrifuged at 13,000 rpm for 15 min, and then the aqueous phase was placed into new sterilized Eppendorf tube.

6. Aliquots of 600 µl isopropanol and 50 µl of sodium acetate (3 M) were added, and then centrifuged at 13,000 rpm for 10 min.
7. The supernatant was discarded.
8. The precipitated DNA was washed with 600 µl of 70% ethanol, centrifuged at 13000 rpm for 5 min, and then the supernatant was discarded.
9. DNA was air dried for 30 min and dissolved in 50 µl of TE buffer.
10. Incubation for 60 min at 65 ºC in a water bath.
11. DNA concentration was measured using Nano-Drop.

2.2.17 Estimation of DNA and RNA concentrations

DNA or RNA concentrations were read with a Nano–Drop spectrophotometer apparatus.

2.2.18 Reagents for gel electrophoresis

1. Agarose.
2. 1X TBE buffer (Tris-borate-EDTA, 0.089 M Tris base, 0.089 M boric acid, 0.002 M EDTA, final pH 8), or 1X TAE buffer.
3. Bromophenol blue in 1% glycerol (loading buffer).
4. Ethidium bromide (10 mg/ml).
5. DNA ladder 100 bp.

2.2.18.1 Preparation of 1% agarose gel (Borges *et al.*, 2009).

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 (2.5 µl) was added to the agarose solution after coolled 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 total RNA, DNA or PCR product.

2.2.18.2 DNA and RNA visualization

1. The first well was loaded with 3 µl of 100 bp of DNA ladder.
2. Each well was loaded with 3 µl of loading dye mixed with 7 µl of total RNA or DNA.
3. PCR product (10 µl) was loaded without adding loading dye (if the dye exists in master mix).
4. The electrical power was turned on after closing the tank lid then adjusted at 80 volt for 1 hr.
5. The migration of DNA was from cathode (-) to anode (+) poles.
6. Agarose gel tray was placed in gel documentation system for visualization of DNA or RNA bands under ultraviolet light (350 nm) and photographed (Couto *et al.,* 2013).

2.2.19 Extraction and purification of total RNA (Geneaid Kit)

Regenerated plantlets which exposed to abiotic stresses and those not exposed to stress (sections 2.2.7.1, 2.2.7.2) were subjected to total RNA isolation using Geneaid total RNA Mini Kit. Glasswares were treated with 0.1% DEPC then autoclaved.

2.2.19.1 Kit components/ 50 samples

1. RB buffer 30 ml
2. PRB buffer 30ml
3. W1 buffer 50ml
4. RNase- free water 6ml
5. Filter column 50 pieces
6. RB column 50 pieces
7. Collection tube 2ml 100 pieces

2.2.19.2 Kit Protocol

Step 1: Tissue Dissociation

Fresh plant tissues were chopped to 50-100 mg, then ground with a mortar and pestle was cooled by pouring a little liquid nitrogen over it and the sample (in liquid nitrogen) was ground to a fine powder, work was carried out quickly with no chance to thaw. The powder was transferred to a 1.5 ml microcentrifuge tube.

Step 2: The Lysis

Aliquot of 500 µl of RB buffer (or PRB buffer) and 5 µl of β-mercaptoethanol were added to the ground sample and mixed by vortex, then incubated for 5 min at room temperature. A filter column was placed in a 2 ml collection tube and the sample mixture was transferred to the column, centrifuged for 1 min at 1000 rpm, then the filtered column was discarded.

Step 3: RNA Binding

Aliquot of 500 µl of absolute ethanol was added to the clarified the mixture filtrates and shacked vigorously. RB column was placed in a 2 ml

collection tube for ethanol transfer – added mixture, then the mixture was centrifuged at 14000-16000 rpm for 2 min.

The flow–through was discarded and RB column was placed back in the 2 ml collection tube then DNase 100 µl was added to the center of the RB column matrix, and left to stand for 10 min.

Step 4: Wash

Aliquots of 400 µl from wash buffer W1was added into the center of the RB column, centrifuged at 14000-16000 rpm for 1 min, the flow – through was discarded and RB column was placed back in the 2 ml collection tube. Wash buffer W2 600 µl was added twice respectively into the center of the RB column, centrifuged at 14000-16000 rpm for 1 min, the flow – through was discarded and RB column was placed back in the 2 ml collection tube, centrifuged at 14000-16000 rpm for 3 min to dry the column matrix.

Step 5 RNA Elution

The dried RB column was placed in 1.5 ml microcentrifuge tube, then RNase- free water 50 µl was added to the center of the column matrix, left to stand for 3 min before centrifugation at 14000-16000 rpm for 3 min (appendix 1).

2.2.20 Synthesis of cDNA (RT-PCR) Reverse transcriptase protocol

Total RNA which extracted from different samples in the above protocol was used as a template to synthesize cDNA by AccuPower® RT Premix. This step included, annealing and reverse transcriptase.

1- Annealing

A primer Oligo–dt was prepared to get 100 pM with DEPC 0.1% and was mixed with template RNA in a sterile tube as indicated below:

Template RNA	0.5 – 1.0 µg	5 µl
Oligo dt_{15} primer	100 pM	5 µl
Reaction Buffer	AccuPower® RT Premix tube	5 µl
DEPC- water		5 µl
Total		20 µl

The annealing mixture (RNA template and Oligo dt_{15} primer) was incubated at 70 ˚C for 5 min and placed on ice.

The incubated mixture was transferred to an AccuPower® RT Premix tube, then filled up the reaction volume with DEPC water.

2- Reverse transcription

The cDNA synthesis was performed using thermocycler reaction as follows:

42 ˚C, for 60 min (cDNA synthesis).

95 ˚C, for 5 min (RTase inactivation).

2.2.21 Polymerase chain reaction (PCR)

2.2.21.1 Random primers

A total of ten random decamer primers (Operon model) were used. Primers in a lyophilized form were dissolved in sterile distilled water to give a final concentration of 100 ρmol, then diluted to a final concentration of 10 ρmol. The primers and their sequences are listed in table (2.2).

2.2.21.2 *AccuPower*®PCR PreMix for random amplified polymorphic DNA RAPD-PCR

Each tube of AccuPower©PCR PreMix was containing 250 µM of each deoxyribonucleoside triphosphate (dNTPs), 30 mM of KCl, 10 mM of Tris- HCl (pH 9.0), 1.5 mM of $MgCl_2$, 1 Unit of Top DNA polymerase and tracking dye.

Table 2.2. Random primers used for amplification of cDNA and genomic DNA (Operon model).

No.	Primer's name	Sequence 5'------ 3'
1	OPA-01	CAGGCCCTTC
2	OPA-05	AGGGGTCTTG
3	OPA-08	GTGACGTAGG
4	OPA-10	GTGATCGCAG
5	OPA-11	CAATCGCCGT
6	OPA-I5	TTCCGAACCC
7	OPA-17	GACCGCTTGT
8	OPB-05	TGCGCCCTTC
9	OPC-04	CCGCATCTAC
10	OPE-08	TCACCACGGT

2.2.21.3 Random amplified polymorphic DNA program polymerase chain reaction (RAPD-PCR) compounds

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

Component	Concentration	Volume (µl)
ddH_2O	______	12.0
AccuPower®PCR PreMix	1X	5.0
Primer	10 pmole	1.0
DNA sample	100 ng/µl	2.0
Total volume		20.0

2.2.21.4 Random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) program

To detect the genetic variations, cDNA was compared with genomic DNA. The following program was adopted.

Step	Temperature (ºC)	Time(min)	No. of cycles
Initial denaturation	94	4	1
Denaturation	94	1	40
Annealing	36	1	
Extension	72	1	
Final extension	72	10	1

The molecular weight of the bands was analyses using the photo Capt MW program.

2.2.22 Analysis of saline drainage water

Total dissolved solids TDS, cations and anions ions in saline water sample were determined in the Chemistry Department, University of Baghdad (table 2.3) and compared with water samples taken from Euphrates river.

Table 2.3. Various parameters measured in saline and Euphrates river water.

Parameter	Unit	Saline water	*Euphrates river water
EC	dS/m^{-1}	120	1.35
T.D.S.	mg/l	431492	-
T.S.S.	mg/l	403576	802
Cl^-	mg/l	87685	139
NO_3^-	mg/l	172.271	2.8
HCO_3^-	mg/l	3708.8	136
$CO_3^=$	mg/l	528	6.25
$SO_4^=$	mg/l	143500	380.75
PO_4^{-3}	mg/l	0.4733	-
Na^+	mg/l	1918	115.6
K^+	mg/l	314	5.7
Ca^{+2}	mg/l	0.0	68.5
Mg^{+2}	mg/l	3700	55
T.H.	mg/l	37500	390
pH	-	8.8	7.6-8.2

*Data in this column are kindly obtained from Prof. M. AL-Dabbas, Geology Dept., Baghdad University.

TDS was calculated according to the followed equation:

TDS (mg/l) = 640 x ECw ($dS.m^{-1}$) (AL-Taee and AL-Humairi, 2013).

2.2.23 Experimented design and statistical analysis

All experiments were designed using Completely Randomized Design (CRD) with 10 replicates except 3 replicates were used in proline experiment. The statistical analysis GenStat software was employed using two way analysis of variance (ANOVA), differences between means were determined and least significant differences were compared at $P \leq 0.05$ (Steel and Torrie, 1982).

Chapter Three

Results and Discussion

3. Results and Discussion

3.1 Sterilization of explants

These experiments were carried out to obtain sterilized explants for *in vitro* cultures.

3.1.1 Sterilization of leaf explants

Surface sterilization with all NaOCl concentrations were significantly better than control treatment as show in table 3.1. The highest leaf explants survival was recorded at 1.5% NaOCl reached 89.4%. All treatments with the disinfectant were not significantly different from each other.

Table 3.1 Effect of (%) NaOCl and duration time on mean leaf explants survival (%), after 28 days on MS medium, n=10.

NaOCl(%)	Time (min)				Mean (%)
	5.0	10.0	15.0	20.0	
0.0	0.0	0.0	0.0	0.0	0.0
0.5	72.0	83.0	93.3	100	87.6
1.0	100.0	100.0	76.7	66.6	85.8
1.5	100.0	100.0	80.0	76.7	89.4
2.0	100.0	83.3	70.3	64.0	79.4
Mean (%)	74.4	73.7	64.1	61.5	
LSD 0.05	NaOCl=15; Time=n.s; Interaction =30				

Although there were no significant differences among duration times but a general reduction trend in explants survival was noticed with increasing the exposure time. The interaction between concentration and duration time was significant for leaf explants survival (%) and ranged from 100% to 0 at 0% NaOCl in all duration times.

3.1.2 Sterilization of stem explants

A gradual increase in survival % at 0.5, 1.0, 1.5 and 2.0 % NaOCL reached 72.1, 76.4, 90.3 and 94.5% respectively compared with control treatment (table 3.2) was recorded. The highest stem explants survival recorded (94.5%) at 2.0% NaOCL, but no significant differences occurred between 1.5 and 2.0% NaOCL treatments. The mean survival % increased with increasing duration times, the highest survival % was recorded at 20.0 min reached 74.4% which was significantly different with survival % at 0.5 min (52.6%) but was not significantly different with the values obtained at 10.0 (69.2%) and 15.0 min (70.6%).

Table 3.2 Effect of NaOCl(%) and duration time on mean stem explants (%) survival, after 28 days of inoculation on MS medium, n=10.

NaOCl(%)	Time (min)				Mean (%)
	5.0	10.0	15.0	20.0	
0.0	0.0	0.0	0.0	0.0	0.0
0.5	43.2	78.2	82.0	85.2	72.1
1.0	49.6	81.4	83.0	96.6	76.4
1.5	80.0	86.4	95.0	100.0	90.3
2.0	90.0	100.0	93.2	95	94.5
Mean (%)	52.6	69.2	70.6	74.4	
LSD 0.05	NaOCl=12.35; Time=11.05; Interaction =24.70				

The interaction effect between NaOCl and duration time on survival was significant. Maximum (100.0) % survival was recorded at 1.5 and 2.0% NaOCl at 20.0 and 10.0 min respectively. Optimization experiments are therefore necessary to achieve maximum survival rate with minimum concentration and exposure time. Sodium hypochlorite (NaOCl) is the main surface sterilizing agent used in plant cell and tissue culture experiments. The effect of NaOCl on explants sterilization is due to the activity of hypochlorous acid (HOCl) which is considered a strong

oxidizing agent released as a result of the reaction between the chlorine compound and water as shown below:

$Cl_2 + H_2O = HCl + HOCl$ (Ramawat, 2008; Colgecen *et al.,* 2011).

3.2 Callus induction

3.2.1 Callus induction on leaf explants

The effect of different concentrations of 2,4-D and Kin on the response to callus induction on leaf explants was shown in table 3.3. Leaf explants responded significantly to the concentration 0.5 mg/l of Kin recording 86.68% however, additional increments in Kin were significantly not affected callus induction. Inclusion of the culture medium with all 2,4-D concentrations caused a significant increase in mean response % for callus induction compared with those cultured on a hormone free medium.

Table 3.3 Effect of 2,4-D and Kin and their interaction on leaf explants response (%) for callus induction, after inoculation on MS medium for four weeks, n=10 (each replicate contain 8 pieces) .

2,4-D (mg/l) / Kin (mg/l)	0.0	0.5	1.0	1.5	2.0	Mean(%)
0.0	0.0	14.9	62.3	71.5	31.3	36
0.5	0.0	83.6	53.3	96.3	63.3	86.68
1.0	0.0	28.0	51.3	75.5	77.3	46.42
1.5	0.0	28.2	30.5	49.0	68.3	35.2
2.0	0.0	16.9	44.6	82.8	78.8	44.62
Mean(%)	0.0	34.32	48.4	75.02	63.8	
LSD 0.05	2,4-D= 12.18; Kin= 12.18; Interaction= 27.25					

The highest response was achieved at 1.5 mg/l 2,4-D reached 75.02%. This value is significantly different with the mean response % at 0.5 and

1.0 mg/l 2,4-D, but is not significantly different with the value obtained at 2.0 mg/l 2,4-D. The interaction between Kin and 2,4-D figure (3.1) revealed that the highest friable yellowish white callus induction (96.3%) was recorded in the treatment 0.5 mg/l Kin +1.5 mg/l 2,4-D followed by the treatment containing 0.5 mg/l Kin + 0.5 mg/l 2,4-D. This result is similar to those obtained by Shabana *et al*. (2001) in *Ruta* who reported a high response for callus induction using this combination.

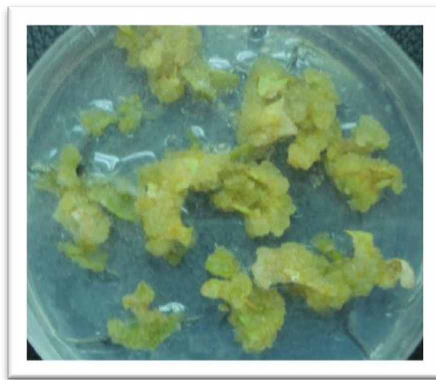


Figure (3.1): Callus culture induced on *R. graveolens* leaf explants grown on MS medium supplemented with 0.5 mg/l Kin and1.5 mg/l 2,4-D after 4 weeks.

3.2.2 Callus induction using stem explants

In order to establish the most suitable concentration of plant growth regulators for callus induction on stem explants, various concentrations and combinations of 2,4-D and Kin were tested. Table 3.4 indicates that a significant increase occurred in the mean % of callus induction at all Kin concentrations compared with 0.0 mg/l Kin (50.4%). The highest value was achieved at 0.5 mg/l Kin recorded 71.64%, which was significantly different with the mean response % at 1.5 and 2.0 mg/l Kin reached 57.8 and 46.8% respectively. A significant differences were recorded in mean % callus induction with increasing 2,4-D concentration at 0.5, 1.0, 1.5 and 2.0 mg/l reached 56.1, 73.84, 79.62 and 80.62% respectively compared with 0.0 mg/l 2,4-D (0.0%). Maximum response was obtained

at 2.0 mg/l 2,4-D. All treatments supplemented with 2,4-D were not significantly different from each other, but significantly different with the value achieved at 0.5 mg/l 2,4-D. Explants showed no response in callus induction when augmented with Kin only.

Table 3.4 Effect of 2,4-D and Kin and their interactions on mean % of response to callus induction, after stem explants culture on MS medium for four weeks, n=10 (each replicate contain 6 pieces).

Kin (mg/l) \ 2,4-D (mg/l)	0.0	0.5	1.0	1.5	2.0	Mean
0.0	0.0	58.0	38.7	88.7	66.9	50.4
0.5	0.0	96.7	95.8	85.7	80.0	71.64
1.0	0.0	35.2	100.0	88.3	82.0	61.1
1.5	0.0	69.8	60.2	86.7	72.3	57.8
2.0	0.0	20.0	68.6	54.6	90.0	46.8
Mean	0.0	56.1	73.84	79.62	80.62	
LSD 0.05	2,4-D =13.39; Kin =13.39; Interaction =29.95					

The highest value of callus induction was achieved at equal concentrations of 1.0 mg/l 2,4-D and Kin recorded 100% figure (3.2) giving rise to a morphogenic friable yellowish white callus. Callus growth obtained in the culture medium supplemented with 1 mg/l of 2,4-D and Kin displayed good biomass production. These results are in agreement with those obtained by Baumert *et al.* (1992) and Shabana *et al.* (2001) who reported that a high callus induction % on *Ruta* hypocotyl and stem explants respectively were induced on MS medium supplemented with equal concentrations of 1 mg/l 2,4-D and Kin.

Initiation of callus using cytokinin alone has not been promising in both leaf and stem explants. This result is also in line with the results of

Arivalagan *et al.* (2012) who reported that callus was not induced in a medium supplemented with Kin only. Callus was efficiently induced on a medium supplemented with 2,4-D only or in combination with Kin on both leaf and stem explants. These results are similar to those obtained by Sakpere *et al*. (2014) who reported that supplementation of 2,4-D in combination with Kin gave the highest cumulative percent of callus induction. Callus of *Ruta* showed a homogenous undifferentiated growth pattern in the presence of 2,4-D and Kin. Leaf explants developed *in vitro* induced a little calli and this due to *Ruta* leaves nature since they are very thin and small in size.

Figure (3.2): Callus induction on *R. graveolens* stem explants using MS medium supplemented with equal concentrations of 1.0 mg/l 2,4-D and Kin after 4 weeks.

Additionally, it may due to the structure of mesophyll tissue that consists of parenchyma cells with enlarged vacuolated cells, rich in intercellular spaces. According to Ziv *et al.*(2008), the leaf cells are surrounded by very thin cell walls and contained a relatively thin cytoplasm, enclosing a large vacuole. Results showed that cells of stem explants in a nutrient medium entered into active division rapidly.

Consequently, calluses of dense consistency were formed possessing a well defined morphogenic capacity which is probably characteristic for this genotype. Addae Frimpomaah *et al.* (2014) and Sakpere *et al*. (2014) reported that the genotype, the type of explant and the concentration of growth regulator 2,4-D play a critical role in callus formation.

3.3 Maintenance of callus cultures

3.3.1 Effect of 2,4-D and Kin (mg/l) on mean callus fresh weight

For callus proliferation, callus initiated on MS medium containing different concentrations of 2,4-D and Kin was aseptically transferred to MS medium with varying concentrations of 2,4-D (0.0 - 2.0 mg/l) and Kin (0.0 - 2.0 mg/l). Results displayed in table 3.5 reveal a significant increase in the callus fresh weight at 0.5, 1.0 and 1.5 mg/l Kin reached 242.0, 62.0 and 82.0 mg respectively while decreased at 2.0 mg/l Kin (47.0 mg) compared with a medium lacking Kin (59.0 mg).

Table 3.5. Callus fresh weight (mg) grown on MS medium supplemented with different combinations of 2,4-D and Kin, after 28 days, the initial callus fresh weight is 50 mg, (n=10).

2,4-D (mg/l) / Kin (mg/l)	0.0	0.5	1.0	1.5	2.0	Mean
0.0	0.0	54.0	62.0	124.0	54.0	59.0
0.5	0.0	205.0	758.0	98.0	150.0	242.0
1.0	0.0	60.0	87.0	64.0	99.0	62.0
1.5	0.0	65.0	79.0	199.0	64.0	82.0
2.0	0.0	0.0	47.0	60.0	126.0	47.0
Mean	0.0	77.0	207.0	109.0	99.0	
LSD 0.05	2,4-D =13; Kin =13; Interaction =29					

The highest callus proliferation value was recorded at 0.5 mg/l Kin. The values at 0.5 and 1.5 mg/l Kin are significantly different with all other calli proliferation values at Kin concentrations.

No significant differences at 1.0 and 2.0 mg/l Kin were reported in a medium lacking Kin. Callus proliferation increased significantly at 0.5, 1.0, 1.5 and 2.0 mg/l 2,4-D reached 77.0, 207.0, 109.0 and 99.0 mg respectively compared with a medium devoid 2,4-D (0.0 mg).

Generally, callus subcultured on MS medium supplemented with 1.0 mg/l 2,4-D and 0.5 mg/l Kin showed better callus proliferation, compact in morphology and yellowish compared to other treatments (Figure 3.3).

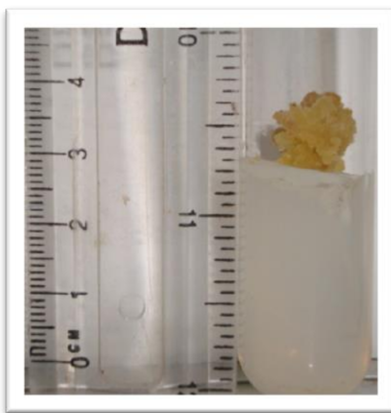


Figure (3.3): *R. graveolens* callus maintained on MS medium supplemented with 1.0 mg/l 2,4-D and 0.5 mg/l Kin after 6 weeks.

The above combination was considered the optimum in terms of high callus production reached 758.0 mg accompanied with the absence of browning. Callus fresh weight increased by 15 fold in the current study, whereas increasing the concentration of 2,4-D to 0.5 or 1 mg/l resulted in 80% and 70% callus proliferation respectively.

A significant increase was recorded in callus fresh weight at 0.5, 1.0, 1.5 and 2.0 mg/l 2,4-D combined with 0.0, 0.5, 1.0, 1.5 and 2.0 mg/l Kin

compared with the control (without 2,4-D) since no callus formation was observed. Nawaz *et al*. (2013) in wheat reported that callus was induced and grew well with further increase in 2,4-D concentrations. Callus mass rate of *Ruta* decreased at 0.5 and 1.0 mg/l 2,4-D interacted with 2.0 mg/l Kin reached 0.0 and 47.0 mg respectively while maintaining equal concentrations of 2,4-D and Kin at 0.5, 1.5 and 2.0 mg/l produced high callus fresh weight reached 205.0, 199.0 and 126.0 mg respectively.

Results showed that calli requires the presence of 2,4-D as auxin for essential induction, this acts as an inductive auxin signal to trigger the proliferative activity of cells. The presence of both 2,4-D and Kin in the medium is necessary for optimum callus formation. Karimi *et al.* (2014) reported that MS medium supplemented with 2,4-D and Kin produced optimum callus formation on hypocotyl and leaf segments in *Satureja* species. Auxin is generally required for the induction of callus from explants. Cells, which respond to auxin, revert to a dedifferentiated state and begin to divide.

Auxin can regulate cell expansion, growth, division and differentiation by accelerating cellular metabolism in treated tissue including leaf, stem and calli. Many of the increased metabolic activities in response to auxin treatment have been attributed to the changes in membrane permeability and activation of some membrane factors. As a consequence of increased respiratory activities, amino acid metabolism, nucleic acid synthesis and protein synthesis and others, cells build up the required materials for their growth. Plant growth involves the interaction between metabolites such as sugars, phytohormones influencing gene expression. The effectiveness of auxin's activity is due to the presence of auxin binding proteins, they act as receptors and after complexing with the auxins, they render highly active (Gaspar *et al.,* 1996). The interaction

between auxin and cytokinin has been interpreted as that auxin plays permissive role in DNA synthesis and cytokinin stimulates it. Cytokinins are concerned in many plant processes, including cell division and shoot morphogenesis (Bressan *et al.*, 2002; Machakova *et al.,* 2008).

3.4 Shoots regeneration from callus cultures

In order to establish the most suitable concentration of plant growth regulators for shoot regeneration, various levels and combinations of BA and NAA were tested. Results exhibited in table 3.6 confirmed the presence of a significant increase in the mean % of shoot regeneration with increasing the concentration of NAA at 0.3, 0.4 and 0.5 mg/l reached 79.9, 74.5 and 96.2% respectively.

Table 3.6. Effect of NAA and BA and their interaction on mean % of shoot regeneration, after inoculating callus pieces for 3 months on MS medium, n=10.

BA(mg/l) / NAA (mg/l)	1.5	2.0	2.5	3.0	Mean
0.3	62.2	90.3	83.1	83.5	79.9
0.4	92.0	79.3	59.7	66.9	74.5
0.5	98.3	96.7	97.3	92.3	96.2
Mean	84.2	88.8	80.0	80.9	
LSD 0.05	NAA =5.45; BA = 6.30; Interaction =10.90				

The highest percentage of shoot regeneration was achieved when NAA at 0.5 mg/l was added to the culture medium, while the highest value of shoot regeneration was obtained at 2.0 mg/l BA reached 88.8%. This value is significantly different with some other values at the concentrations 1.5, 2.5 and 3.0 mg/l BA, but these later treatments were

not significantly different from each other. Interaction between BA and NAA caused significant shoot regeneration values was recorded at 1.5, 2.0, 2.5 and 3.0 mg/l BA after the inclusion of NAA at 0.5 mg/l reached 98.3, 96.7, 97.3 and 92.2% respectively.

The yellow friable callus became more greenish and appeared highly competent for shoot initiation (figure 3.4 A,B) when transferred to a medium containing different concentrations of BA and NAA, particularly on a medium enriched with 1.5 mg/l BA and 0.5 mg/l NAA, (figure 3.4 C,D). It recorded high frequency of shoot regeneration (98.3%). These results are in accordance with those showed by Ahmed *et al.* (2010) in *Ruta* who reported high tendency of callus to give rise multiple shoots (92.3%) on MS medium supplemented with 1.68 mg/l BA and 0.186 mg/l NAA.

No significant differences were recorded among the treatments 1.5, 2.0, 2.5 and 3.0 mg/l of BA in the presence of NAA at 0.5 mg/l, since these concentrations recorded the highest values of shoots regeneration. Gurudeeban *et al.* (2011) revealed that best frequency for shoot induction in *Ruta* was obtained on MS medium containing 2.5 and 0.5 mg/l BA. BA as a cytokinin is an essential for cell division, although in the current study relatively high levels of BA (1.5-3.0 mg/l) was required for shoot regeneration, Zuraida *et al.* (2014) reported that much lower BA 0.5 mg/l for *R. graveolens* shoot regeneration. While Bohidar *et al.* (2008) demonstrated that the highest number of *Ruta* shoot buds was obtained on MS medium containing 1.0 mg/l BA and 0.25 mg/l IAA.

Mok and Mok, (2001) reported that the inclusion of cytokinins is

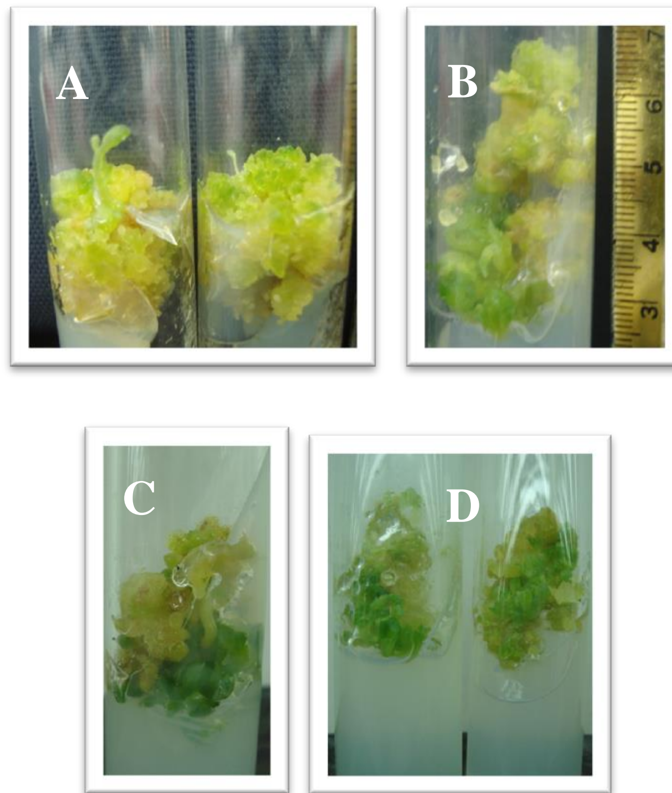


Figure (3.4): (A, B) Cultures showing shoot bud emergence from *R. graveolens* callus grown on MS medium supplemented with 1.5 mg/l BA and 0.5 mg/l NAA after 6 weeks. (C, D) Induction of shoot buds and primary leaves on the same medium after 12 weeks.

important since these growth regulators, such as BA, promote cell division and plant growth and development. Cytokinins play multiple roles in the control of plant development, they stimulate protein synthesis and participate in cell cycle control and promote the maturation of chloroplasts. The effect of cytokinins is most noticeable in tissue cultures where they are commonly used, often accompanied with auxins, to stimulate cell division and control morphogenesis.

They play multiple roles in the control of plant development by activating RNA synthesis, stimulate protein synthesis and some enzyme activities (Machakova *et al.,* 2008). Cytokinin concentration has been reported to be a decisive for shoot proliferation and elongation in many

plants species. Clearly, the low concentration of NAA showed a synergistic effect with BA and increased the shoot morphogenic response. Similar observation with auxin and cytokinin combination has been recorded by Faisal *et al*. (2005) who found high shoot regeneration frequency (98.5%) initiated form *Ruta* calli on MS medium supplemented with 2.25 mg/l BA and 0.46 mg/l NAA.

3.5 Elongation of multiple shoots

Clumps of multiple shoots reported in 3.4 were transferred to MS medium enriched with various concentrations of GA_3 for further shoot proliferation and elongation, already formed (table 3.7). Results show high value of shoots length was achieved at 0.5 mg/l GA_3 reached 2.18 cm. This value is significantly different with mean shoot length of all other treatments at 0.0, 0.1 and 1.0 mg/l of GA_3. Maximum number of shoots was recorded (29.3) at 0.5 mg/l GA_3, and this value is significantly different with mean shoots number at 1.0 mg/l GA_3 only. All other treatments were not significantly different from each other.

Table 3.7 Effect of different GA_3 concentrations (mg/l) on mean number of shoots and length (cm), after transfer for 6 weeks on MS medium, n=10

GA_3 (mg/l)	Mean no. of shoots	Mean of shoots length cm
0.0	19.3	0.92
0.1	20	1.16
0.5	29.3	2.18
1.0	10	0.93
LSD 0.05	17.19	1.103

Data indicated that GA3 promoted proliferation and elongation of shoots as shown in figure 3.5 (A,B). These results are in line with those obtained by Ndagijimana *et al.* (2014) in potato who reported that MS

medium supplemented with 0.5 mg/l GA_3 produced the longest shoots. The optimum level of GA_3 that promoted the highest mean number of multiple shoots was 0.5 mg/l, and these results are in accordance with those obtained by Kalyani and Rao (2014) in tomato who found that MS medium supplemented with 0.6 mg/l GA_3 showed maximum percentage of shoot elongation. While Srivastarva and Raghav (2014) reported that 1.0 mg/l GA_3 was the best concentration since it induced the highest number of shoots. Gibberellin increases both cell elongation and cell division, as evidenced by increases in cell length and cell number by increasing cell division activity in the meristemetic cells in response to the applications of gibberellins (Bressan *et al.,* 2002; Moshkov *et al.,* 2008).

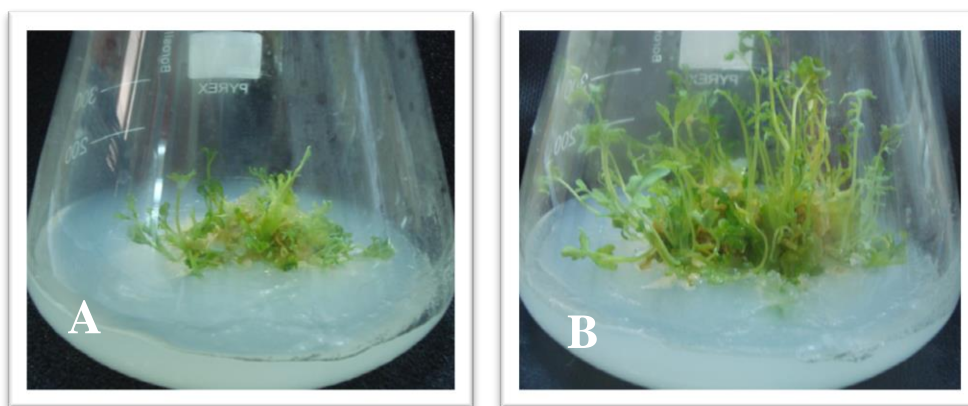


Figure (3.5): (A) Multiple shoots of *R.graveolens* transferred on MS medium supplemented with 0.5 mg/l GA_3 after 4 weeks. (B) Shoot elongation on MS medium supplemented with 0.5 mg/l GA_3 after 6 weeks.

3.6 Rooting

Production of plantlets with profuse rooting *in vitro* is important for successful establishment of regenerated plantlets. Shoots were transferred onto a half strength MS medium supplemented with 0.1 mg/l IBA for rooting. The rooting of shoots success percentage was 100% for shoots

regenerated from callus cultures (fig 3.6 A, B). These results are in agreement with those obtained by Faisal *et al*. (2005) in *Ruta* who reported that *in vitro* regenerated shoots rooted better on a half strength MS medium containing 0.1 mg/l IBA. Also Ahmed *et al.* (2010) demonstrated that regenerated shoots of *R. graveolens* were rooted *in vitro* on MS medium containing 0.1mg/l IBA.

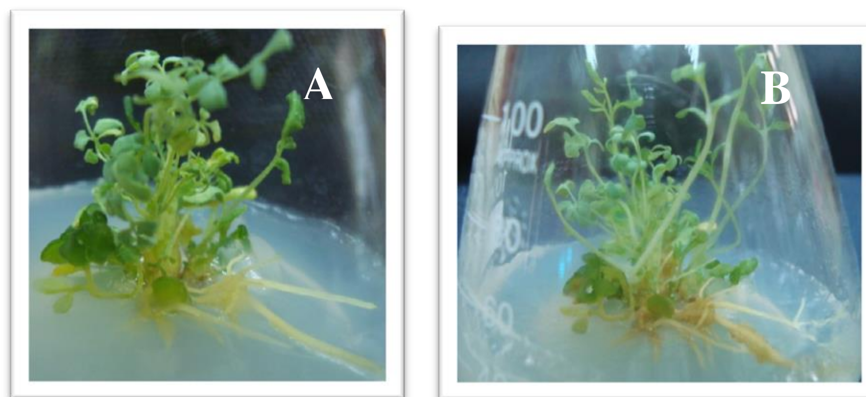


Figure (3.6): Rooting regenerated shoots of *R. graveolens* callus cultures transferred onto $^{1}/_{2}$ MS medium supplemented with 0.1 mg/l IBA. (A, B) after 6 weeks.

While Bohidar *et al*. (2008) found that elongated shoots were rooted on a half strength MS medium supplemented with 0.5 mg/l IBA. Auxins promote root initiation by stimulates cells to divide. Auxin also induces both growth of pre-existing roots and adventitious root formation (Taiz and Zeiger, 2002).

3.7 Screening of callus cultures on different abiotic stresses

In vitro selection for tolerance to abiotic stresses depends on the development of efficient and reliable callus induction and plant regeneration systems. Hence these experiments were carried out for

screening *R. greaveolens* calli against mannitol and saline water, then evaluating the effects of these stresses at the cellular level.

3.7.1.1 Direct screening method for callus cultures

In vitro callus cultures were exposed directly to different concentrations of mannitol (0.0 – 300 g/l). Results (figure 3.7) revealed that increasing water stress as induced by mannitol caused a reduction in callus fresh weight. A significant decrease occurred in the mean of callus fresh weight at 60,120,180, 240 and 300 g/l mannitol reached 158, 119, 86.7, 142.6 and 91.7 mg respectively as shown in figure 3.8 compared with a mannitol free medium 206 mg.

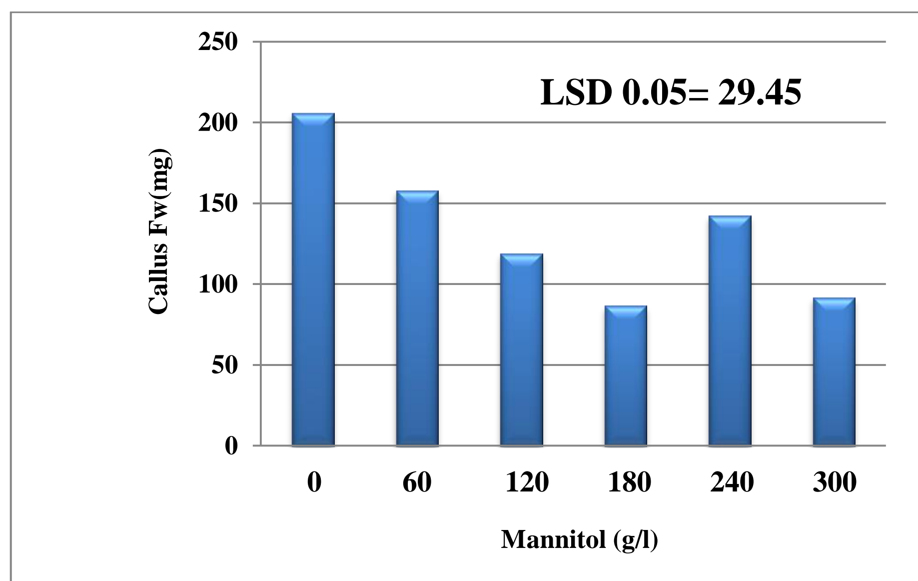


Figure (3.7): Effect of mannitol concentration on mean callus fresh weight (mg) after inoculation of callus pieces were inoculated onto a solid MS medium supplemented with 1mg/l of 2,4-D and 0.5 mg/l of Kin after four weeks by using direct method of screening for drought tolerance. Initial weight is 100 mg, n=10.

Maximum callus fresh weight reached 158 mg at 60 g/l mannitol. This value is significantly different with mean callus fresh weight of all other treatments except 240 g/l. The value recorded at 240 g/l was significantly different with treatments at 180 and 300 g/l, while the value recorded at

120 g/l was significantly different with the value at 180 g/l only. A decrease in callus growth is a typical response of calli to mannitol and PEG stresses (Matheka *et al*., 2008).

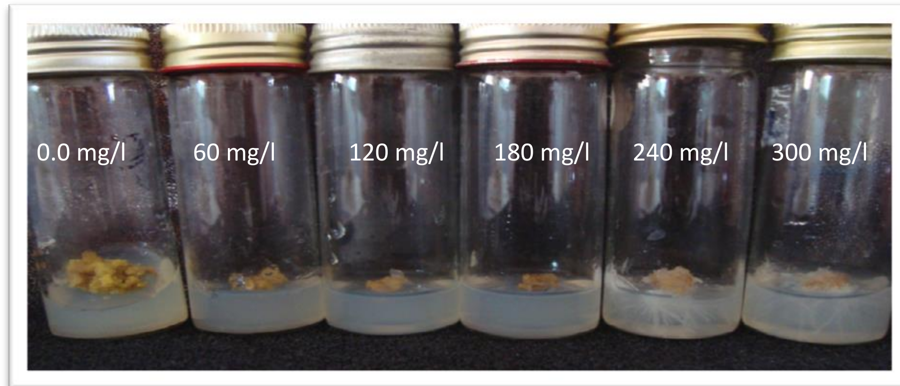


Figure (3.8): *Ruta graveolens* callus cultures exposed to different concentrations of mannitol showing changes in callus mass grown on MS medium supplemented with 2,4-D 1mg/l and Kin 0.5 mg/l, with different concentrations of mannitol for four weeks.

The above results are in line with those obtained by Haque *et al.* (2013) in rice who found a reduction in callus fresh weight with increasing levels of mannitol stress. Data displayed high increase occurred in the mean callus fresh weight at 240 g/l mannitol reached 142.6 mg, these results are better than those obtained by Wani *et al.* (2010) who observed that the capacity of rice callus induction developed on MS medium decreased when cultures exposed to 16 mg/l PEG. The reduction in water content caused a decrease in cell turgor pressure and consequently reduction in callus growth as expressed as callus fresh weight (AL-Taha, 2013).

Callus growth reduction is probably due to the reduction of cytoplasmic and vacuolar volume resulted from removal of water from cytoplasm by lowering cellular water potential. A lowered external osmotic potential is detrimental as cells unable to take up water and nutrients from the external environment leading to a decline in cell

growth (Matheka *et al.,* 2008; Cazares *et al*., 2011). A significant increase in callus growth was recorded in response to 240 g/l mannitol after twelve weeks (figure 3.9) reached 290, 349 and 568 mg respectively after 4, 8 and 12 weeks to a medium lacking mannitol. Callus fresh weight under water stress (240 g/l mannitol) reached 112, 158 and 188 mg for the three periods respectively. It seems that callus growth increased slowly with increasing the time of exposure to mannitol. The highest callus fresh weight was recorded at 240 g/l mannitol reached 188 mg after 12 weeks.

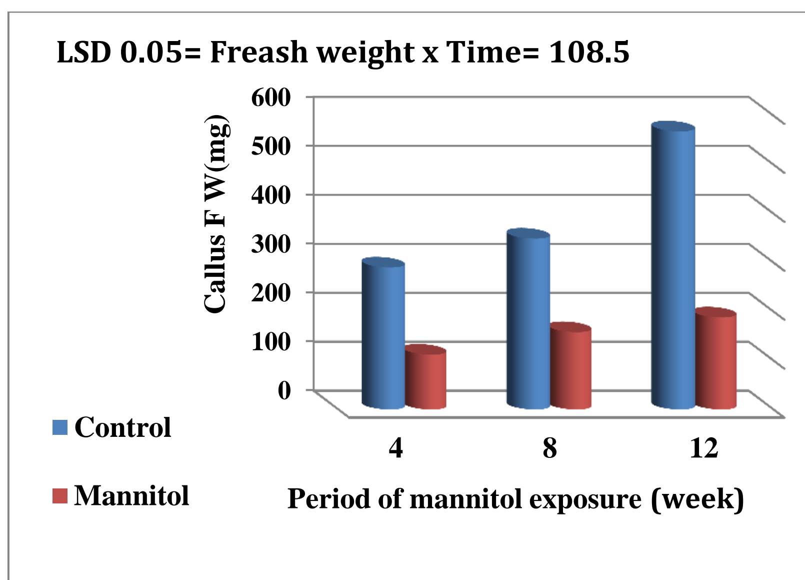


Figure 3.9: Effect of the time course on *R. graveolens* callus fresh weight supplemented with 240 g/l of mannitol, callus pieces inoculated onto a solid MS medium supplemented with 1mg/l of 2,4-D and 0.5 mg/l of Kin for 4, 8 and 12 weeks. Initial weight is 100 mg, n=10.

This value is not significantly different with the mean callus fresh weight at 240 g/l mannitol after 4 and 8 weeks. Abdel-Raheem *et al.* (2007) reported that growth dynamic of tomato callus culture deceased significantly with increasing the exposure period of mannitol and PEG. Mohamed and Ibrahim (2012) suggested that mannitol is taken up and metabolized by plants as an alternative carbon source, but increasing the carbohydrate concentrations in the growth medium up to a limit,

increases the water potential, resulting in reduced water uptake and thus causing growth inhibition. Water stress determines cell division and elongation causing growth reduction (AL-Bahrany, 2002). Stress induce osmotic shock due to reduced cell division, shrinking imbalance due to a reduced loss of cell turgor, nutritional imbalance as a result of reduced water uptake, an increase in electrolyte leakage and a decrease in cell water contents with increasing stress (Lokhande *et al*., 2010; Wani *et al*., 2010).

3.7.1.2 Stepwise screening method of callus cultures on different mannitol concentrations

In vitro callus cultures were exposed to gradual increments in mannitol (0.0–300.0 g/l). Data (fig 3.10) show no significant differences in callus fresh weight between untreated calli and those exposed to 60 and 120 g/l mannitol. A significant decrease in callus fresh weight in a mannitol free medium and 180, 240 and 300 g/l mannitol reached 428, 490 and 578 mg and 278, 277 and 281 mg respectively for gradual treatment. Inclusion of the culture medium with all mannitol concentrations caused no significant differences in mean callus fresh weight between treatments.

Maximum callus fresh weight was recorded at 300 g/l mannitol reached 281 mg after the 5th re-culture. Results exhibited that gradual exposure to mannitol has no clear decline in callus growth. These results are in line with those obtained by Mitoi *et al.* (2009) who revealed that long term exposure to mannitol at high concentrations, may activate the adaptive mechanisms by which the expression of antioxidant enzymes are correlated. Hadi *et al.* (2014) stated that plants respond to drought stress in very intricate and diverse way, and it is impractical to consider a single gene is responsible for drought tolerance.

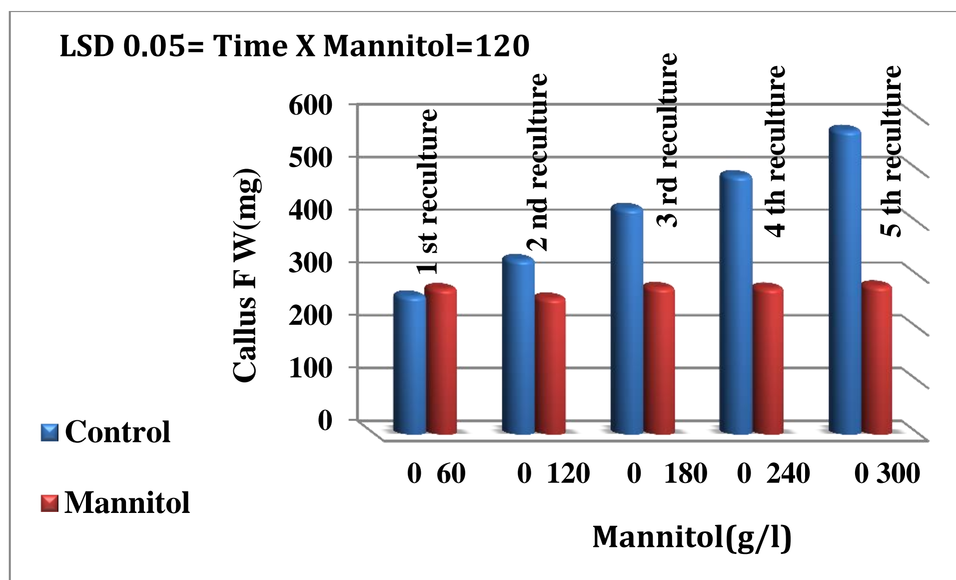


Figure 3.10. Effect of mannitol on mean callus fresh weight after inoculation of callus pieces onto a solid MS medium supplemented with 1mg/l of 2,4-D and 0.5 mg/l of Kin for twenty weeks using stepwise increments of mannitol concentrations at four week intervals. Initial weight is 100 mg, n=10.

The gradual exposure of callus to mannitol induces high expression for a number of genes like osmo–proteins (shock proteins), but these proteins do not form when calli are exposed to shock treatment using mannitol (Ziegler, 1990). The over expression of these genes activate the expression of many downstream genes thus plants express improved stress tolerance (Peleg *et al.,* 2011). Leone *et al.* (1994) revealed that protein synthesis was not inhibited by gradual stress imposition, and the expression of many proteins was induced in adapted cells, also he suggested that plant cells are able to monitor different levels of stress intensity and modulate gene expression accordingly. These results are also in line with those reported by Abdul-Baker (2012) in tomato and AL-Taha (2013) in sour orange who concluded that stepwise treatments are better than shock ones.

3.7.2.1 Direct screening method for callus cultures on different saline water concentrations

In vitro callus cultures were exposed to direct concentrations of saline water (5.0 – 30.0 $dS.m^{-1}$). Results (figure 3.11) revealed that increasing stress as induced by saline water caused a reduction in callus fresh weight except at 10 $dS.m^{-1}$.

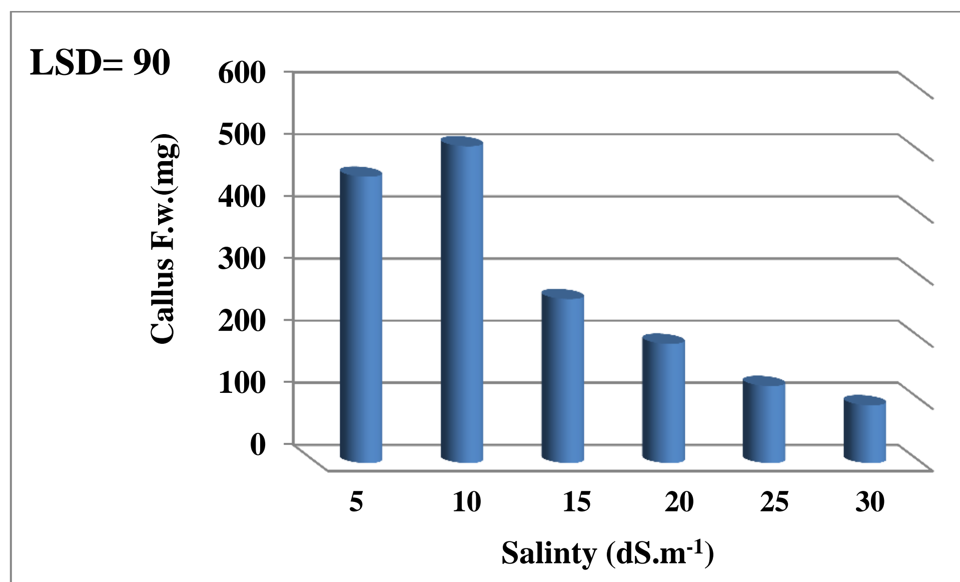


Figure (3.11): Effect of saline water concentrations on mean callus fresh weight of *R. graveolens* after inoculation of callus pieces onto a solid MS medium supplemented with 1mg/l of 2,4-D and 0.5 mg/l of Kin after four weeks by using direct method of screening for salt tolerance. Initial weight is 100 mg, n=10.

A significant decrease occurred in the mean callus fresh weight at 15, 20, 25 and 30 $dS.m^{-1}$ of saline water reached 264, 192, 124 and 93 mg respectively compared with the control at 5.0 $dS.m^{-1}$(461 mg) for shock exposure (figure 3.12). These results are corroborate with those of Panghal and Soni (2013) who obtained a decrease in callus fresh weight in *Jatropha curcas* callus cultures when subjected to increasing concentrations of salt in MS medium. The highest value for callus fresh weight obtained at 10 $dS.m^{-1}$ of saline water reached 503 mg. This value

is significantly different with all stress treatments, but is not significantly different with control at 5.0 $dS.m^{-1}$. The morphology and growth of the tolerant calli at 10.0 $dS.m^{-1}$ were similar to the control which maintained at 5.0 $dS.m^{-1}$. Similarly, Taha (2014) reported that increasing seawater levels in the proliferation medium increased Jojoba callus fresh weight. The value for callus fresh weight at 15 $dS.m^{-1}$ is significantly different with the values recorded at 25 and 30 $dS.m^{-1}$ only, while no significant difference between the values recorded at 20, 25 and 30 $dS.m^{-1}$.

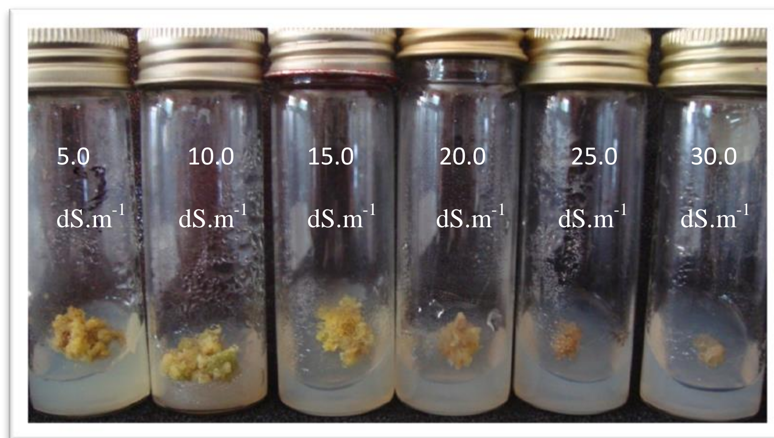


Figure (3.12): *Ruta graveolens* callus cultures exposed to different concentrations of saline water showing changes in callus mass grown on MS medium supplemented with 2,4-D 1mg/l and Kin 0.5 mg/l, for four weeks.

Rao and Patail (2012) cultured bean callus tissues on a medium containing 2.9 mg/l NaCl. Cultures showed good cell proliferation and appeared morphologically similar to control. The callus fresh weight decreased gradually with increased the concentration of saline water in the culture medium. These results are in line with those reported by Taha and Hussan (2014) who demonstrated that date palm callus cultures exhibit an increase in callus fresh weight at low levels of seawater, but lowest callus fresh weight was produced with increasing sea water concentration in the medium. Bekheet *et al.* (2006) reported that callus

fresh weight of onion decreased as salt mixture is raised in the culture medium. Marroquin *et al*. (2011) revealed that MS medium supplemented with 4 mg/l NaCl greatly increased the growth and development of pepper somatic embryos, but supplementation of 17 mg/l NaCI to the medium delayed the growth and development of somatic embryos. Salinity inhibits the growth of plants by affecting both water absorption and biochemical processes, such as nitrogen assimilation and protein biosynthesis (Dubey, 1994).

The growth inhibition under osmotic conditions might be mainly due to decreased in cell volume leading to reduction in cytoplasmic volume and the loss of cell turgor as a result of osmotic outflow of intracellular water that may lead to retraction of the plasma membrane from the cell wall (Chinausamy *et al*., 2005; Munns and Tester, 2008). Also Raveendar *et al.* (2008) revealed that increasing salinity levels in MS medium showed inhibitory effect on rice calli initiation.

A significant increase in callus growth was recorded in response to 25 $dS.m^{-1}$ of saline water after twelve weeks (figure 3.13) reached 235, 338 and 536 mg respectively after 4, 8 and 12 weeks for control medium at 5.0 $dS.m^{-1}$. While callus growth under the salinity stress 25 $dS.m^{-1}$ of saline water reached 128, 227 and 233 mg respectively. The callus growth increased markedly by increasing salinity stress at 4 and 8 weeks, but at 12 weeks the callus growth slowed down. Lower callus growth value at 25 $dS.m^{-1}$ of saline water after 4 weeks was significantly different with other treatments at duration time 8 and 12 weeks, and also with control at 5.0 $dS.m^{-1}$ saline water after 4 weeks. The highest callus growth was recorded at 25 $dS.m^{-1}$ of saline water after 12 weeks reached 233 mg. This value was not significantly different with the value obtained at 25 $dS.m^{-1}$ of saline water after 8 weeks but these values were

significantly different with the values of control at 5 $dS.m^{-1}$ saline water after 8 and 12 weeks.

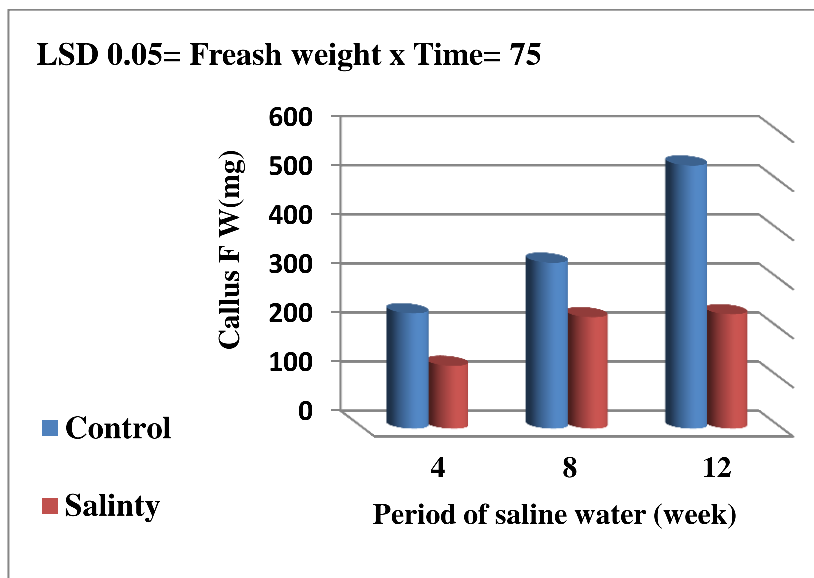


Figure (3.13): Effect of the time course on *Ruta graveolens* callus fresh weight supplemented with 25 $dS.m^{-1}$ of saline water, callus pieces inoculated onto a solid MS medium supplemented with 1mg/l of 2,4-D and 0.5 mg/l of Kin for 4, 8 and 12 weeks. Initial weight is 100 mg, n=10.

These results are better than those revealed by Koc *et al*. (2009) who reported that the *Citrus aurantium* callus growth decreased markedly and discoloration with increasing NaCl at 17.4 mg/l after 12 weeks. Adverse effects of salinity on plant growth may be due to ion cytotoxicity, (Mainly due to Na^{+}, Cl^{-} and $SO4^{-}$), osmotic stress, nutritional deficiency, oxidative stress and hormonal imbalance (Chinausamy *et al*., 2005; Munns and Tester, 2008). Mehdi and Idris (2013) reported that increasing salinity level reduced survival rate and callus growth in tomato. Abbas *et al*. (2012) demonstrated that increasing NaCl concentration up to 18.5 mg/l in MS medium decreased the fresh weight for both embryogenic callus and somatic embryos of date palm after 8 week. The slowdown in growth of callus when the culture period was prolonged beyond 8 to 12

weeks may due to salinity that affected all the morphological parameters and decreased growth performance (Abdul-Jaleel *et al*., 2008).

These results are also better than those reported by Rao and Patil (2012) who revealed that calli of bean died when cultured directly on MS medium supplemented with 11 mg/l NaCl after 4 weeks. High salt stress disrupts homeostasis in water potential and ion distribution leading to molecular damage, growth arrest and even death of whole tissue (Zhu, 2001). Cicek and Cakirlar, (2002) reported that negative effect of salinity on plant growth and water content may be due to the defect in plant cells metabolism. High osmotic pressure resulted from high salinity restricted plant cells to uptake water and some mineral nutrients dissolved in the culture medium. Musznska *et al.* (2014) confirmed that the permeability of water through plasma membrane is disturbed under salt stress. The simple increase in fresh weight at 25 $dS.m^{-1}$ of saline water after 12 weeks may return to restrict salt the uptake and cells adjusted their osmotic pressure in callus tissues, through the synthesis of compatible solutes like proline, glycinebetaine and soluble sugars. Or cells sequester and accumulate salt into the vacuoles in salt-tolerant plants, as stated by Amudha and Balasubramani (2011). Salinity and drought affect many physiological processes but reduced cell growth, leaf area, biomass and yield are the most important ones, reduction in growth is a common phenomenon of salt stressed plants, which has also been observed in cultured cells, tissues or organs on a medium supplemented with NaCl (Rai *et al.,* 2011; Perez and Gomez, 2012).

3.7.2.2 Stepwise screening method for callus cultures on different saline water concentrations

In vitro callus cultures were exposed gradually to different concentrations of saline water (5.0 – 30.0 $dS.m^{-1}$). Results exhibited

that gradual exposure (figure 3.14) showed a clear increase in callus fresh weight. No significant differences in callus fresh weight between control treatment and 10, 15, 20, 25 and 30 $dS.m^{-1}$ of saline water which recorded 257, 354, 530, 629 and 717 mg respectively for control treatment at 5 $dS.m^{-1}$ saline water, while 304, 556, 637, 649 and 669 mg respectively for salinity exposure after twenty weeks. The value 304 mg of callus fresh weight that induced at 10 $dS.m^{-1}$ saline water was significantly different with all salinity treatments except that exposed to 15 $dS.m^{-1}$ saline water, but they were not significantly different between each other. Maximum value was achieved at 30 $dS.m^{-1}$ saline water reached 669 mg after twenty weeks.

These results are in line with those obtained by Queiros *et al.* (2007) who reported that the gradual selection process was more suitable and efficient than the direct one to establish salt tolerant potato calli. A similar response was described in salt-tolerant rice plants established *in vitro* by using the stepwise increase in NaCl treatments (Miki *et al.,* 2001). There is an evidence that gradual exposure to increasing intensity of stress-inducing factor allows physiological and biochemical adjustments, which are the basis for a new cellular homeostasis compatible with the imposed stress (Leone *et al*., 1994).

The current study report better calli performance than those reported by EL-Meleigy *et al*. (2004) who studied callus fresh weight of tomato subjected to a gradual increase in NaCl. There are many strategies used by plant cells for achieving greater tolerance and to re-establish homeostasis in stressful environments. Both ionic and osmotic homeostasis must be restored (Zhu, 2001). Rai *et al*. 2011 reported that salinity causes ion-specific stresses resulting from altered K^+/Na^+ ratios leads to build up in Na^+ and Cl^- concentrations that are detrimental to

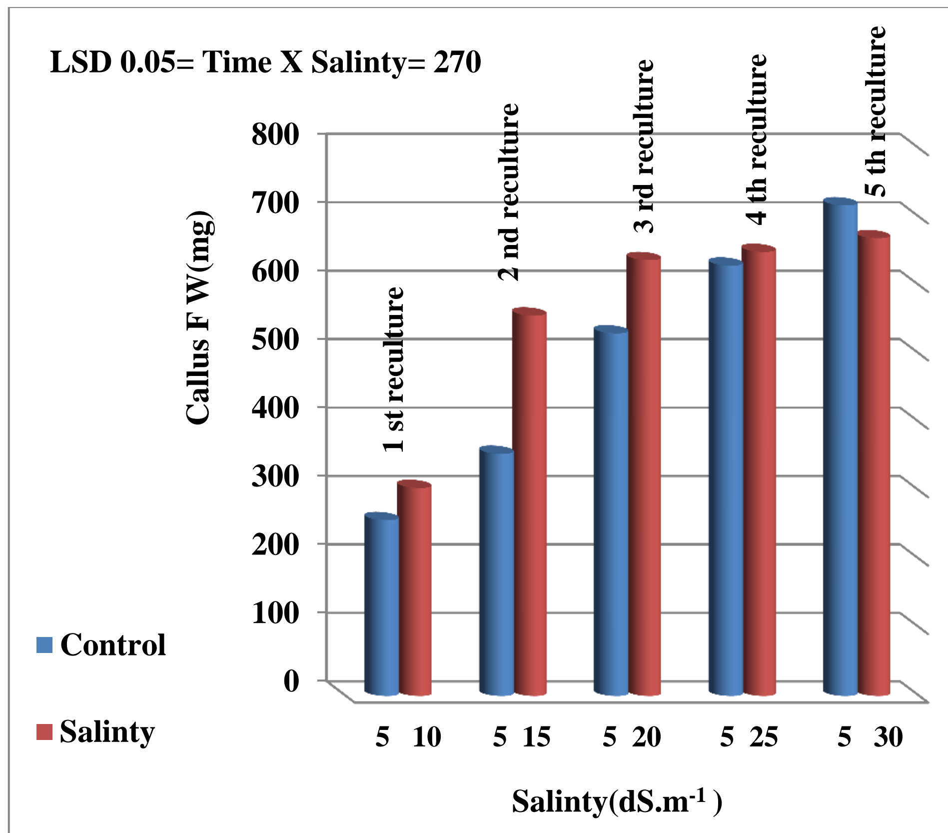


Figure (3.14): Effect of saline water concentrations on mean callus fresh weight after inoculation of callus pieces onto a solid MS medium supplemented with 1mg/l of 2,4-D and 0.5 mg/l of Kin for twenty weeks using stepwise increments of saline water concentrations at four week intervals. Initial weight is 100 mg, n=10.

plants growth. Because intracellular K^+ and Na^+ homeostasis is important for the activities of many cytosolic enzymes, maintaining membrane potential, and for the synthesis of osmoticum (Waditee *et al.,* 2006). Zhu, (2001) reported that preventing high levels of Na^+ accumulating in the cytoplasm or in organelles other than the vacuole is very important for cell growth, because this could result in toxic levels of Na^+ as well as insufficient K^+ concentration for enzymatic reactions and osmotic adjustment and thus suppressing cell growth and development (Waditee *et al.,* 2006; Amudha and Balasubramani, 2011).

On the other hand, Amudha and Balasubramani (2011) and Rai *et al.* (2011) concluded that alteration of ion ratios in plants could result from the influx of Na^+ through pathways that also function in the uptake of K^+, maintenance of a high cytosolic K^+/Na^+ ratio is a key requirement for plant growth under high concentrations of salt. Maintaining a high K^+/Na^+ ratio in the cytosol causes osmotic balance for proper function of the cell, by diminishing the entry of Na^+ ions into the cells or extrusion of Na^+ ions out of the cell and vacuolar compartmentation of Na^+ ions (Waditee *et al.,* 2006; Rai *et al*., 2011 and Lokhande and Suprasanna, 2012).

3.8 Regeneration of callus cultures subjected to abiotic stresses

Calli clumps reported in 3.7.1.1, 3.7.1.2, 3.7.2.1 and 3.7.2.2 were transferred to MS medium enriched with 1.5 mg/l BA and 0.5 mg/l NAA for shoot regeneration. Results displayed in figure (3.15) revealed that a significant difference in the mean % of shoots regeneration occurred in treatments subjected to all salinity and drought stresses. A significant decrease in mean % of shoot regeneration reached 79.93 and 85.03% at 240 and 300 g/l mannitol in both direct and gradual exposure respectively compared with 98.33% regeneration in a medium devoid the stress agents. The highest value for shoot regeneration was recorded after exposure of callus cultures gradually up to 300 g/l mannitol reached 85.03%.

Also a significant decrease in mean number of shoots was recorded (69.63 and 64.13%) at 25 and 30 $dS.m^{-1}$ saline water for direct and gradual exposure respectively compared with 98.33% for non-stressed medium. The direct expose to salinity recorded the highest number of shoots reached 64.31%. Drought tolerant calli using stepwise screening method on mannitol resulted in higher number of shoots. Bouiamrine and Diouri, (2012) reported that calli under high osmotic pressure were able

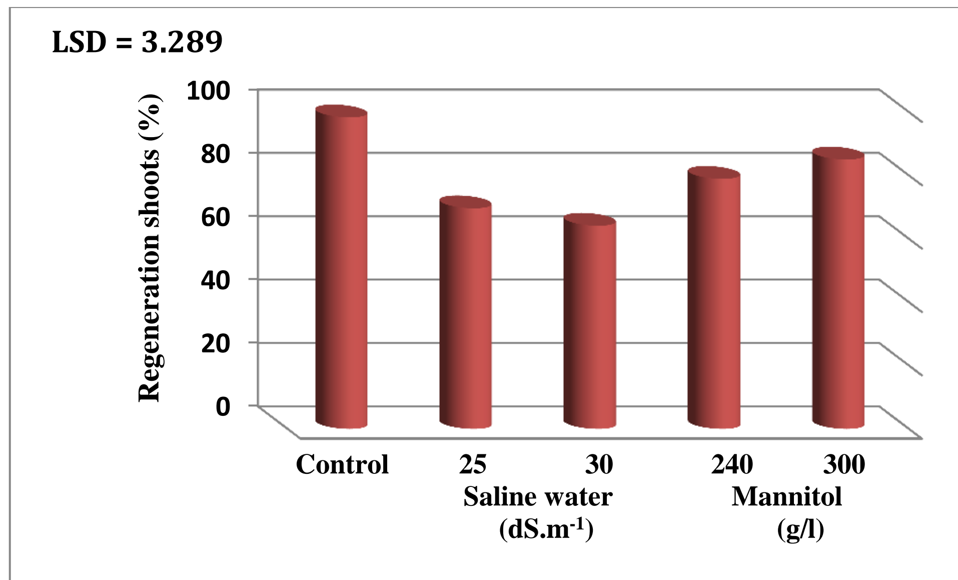


Figure (3.15): Effect of saline water (dS.m^{-1}) and mannitol (g/l) on the mean no. of regenerated shoots/piece, after inoculating callus pieces onto a solid MS medium supplemented with 1.5 mg/l of BA and 0.5 mg/l of NAA for 3 months, n=10.

to adapt against stress which created in the medium by developing new and non- necrotic secondary calli. These calli continued growing in the regeneration medium. The long-term culture of these calli, which seems to tolerate high osmotic pressures and had a good potential for regeneration, made it possible to select tolerant cell lines to water stress. Similar results for direct treatment were reported by Wani *et al.* (2010) in rice who indicated that increasing PEG in the culture medium caused a decrease in callus morphogenic capacity and with Mahmood *et al.* (2012) in wheat, while Matheka *et al.* (2008) reported that shoots regeneration from callus under water stress is inversely proportional to PEG concentration in the selection medium.

This decrease in regeneration capacity resulted under stress may be explained by slowing down all cell physiological processes (Bouiamrine and Diouri, 2012). In contrast, results for gradual exposure reported by Mitoi *et al.* (2009) who indicated that cell cultures exposed to a mild

osmotic stress in a medium supplemented with mannitol may not activate the adaptive mechanism. On the other side, raising the exposure time to mannitol allows tolerant cell lines to grow and suppresses the sensitive ones. The data of % shoot regeneration from callus pieces after employment of high concentrations of mannitol (240 and 300 g/l) for shock and gradual exposure respectively are considered highly optimistic compared with the results of Sakthivelu *et al.* (2008) who reported that a decrease in the total number of viable soybean plantlets regenerated from cotyledonary nodes at 60 g/l PEG.

Plants respond to the stresses at both molecular and cellular levels (Peleg *et al.,* 2011). Expression of a variety of genes has been demonstrated to be induced by these stresses, the products of such genes are thought to function not only in stress tolerance but also in regulation of other gene expressions and signal transduction as a stress response. Genes being induced during drought stress conditions are thought to protect plants from water deficit by producing important metabolic proteins and the regulation of genes for signal transduction as stress response (Vazquez and Linacero, 2010).

It has been previously demonstrated by many workers that incorporation of salt into the medium, generally reduces or even inhibits the regeneration completely. Aazami *et al*. (2010) demonstrated that number of shoot formation in tomato decreases with increasing NaCl concentrations. Likewise, Bekheet *et al.* (2006) also found similar results in onion. These results also in line with the previous studies of Hassanein (2004), Htwe *et al*. (2011), Rao and Patil (2012) and Panghal and Soni (2014) who reported that shoot regeneration was recorded when callus exposed to 4.6 mg/l NaCl, while 5.8 mg/l NaCl induced highly necrotic callus. Shankdhar *et al*. (2000) mentioned that callus cultures may loss the regeneration potential during the long periods of selection.

It has been interpreted that a certain amount of the total energy available for tissue metabolism is channeled to resist the stress. Under stress conditions, one of the strategies is slowing down cell growth and metabolism (Zhu, 2001). The gradual increase of saline water up to 30 $dS.m^{-1}$ with long exposure time (twenty weeks) caused high accumulation of Na^+ and Cl^-. Their injurious effects progresses exponentially with increasing salinity levels. High levels of Na^+ inside the cells inhibits K^+ uptake and as a result it causes an increase in the Na^+/K^+ ratio. This increase might be attributed to the fact that Na^+ causes a disturbance in the ion balance in plant cells by increasing Na^+ uptake.

Many of the deleterious effects of Na^+ seem to be related to the structural and functional integrity of membranes (Cicek and Cakirlar, 2002; Munns and Tester, 2008). Sharma *et al.* (2013) demonstrated that inverse relationship was observed between salt exposure and rough lemon seedlings growth after increased Na^+ and Cl^- levels and a decrease in K^+/Na^+ ratio. Salinity level at 25 $dS.m^{-1}$ with long duration time (twelve weeks) established sustainable growth of calli resulted an efficient regeneration potential (figure 3.16). To combat stress effect, plants develop various biochemical and physiological mechanisms at the cellular and molecular levels to adapt to these stresses and thus acquire stress tolerance (Madhava, 2006; Perez and Gomez, 2012).

Stress at cellular level responses include changes in cell cycle, cell division, endomembrane system, vacuolization of cells and cell wall architecture, all leading to enhanced stress tolerance of cell. At the biochemical level, plants alter metabolism in various ways by induction of antioxidant enzymes, ion homeostasis, tolerance to osmotic stress, synthesis of compatible organic solutes and tissue tolerance (Carillo *et al*., 2011; Rai *et al.,* 2011).

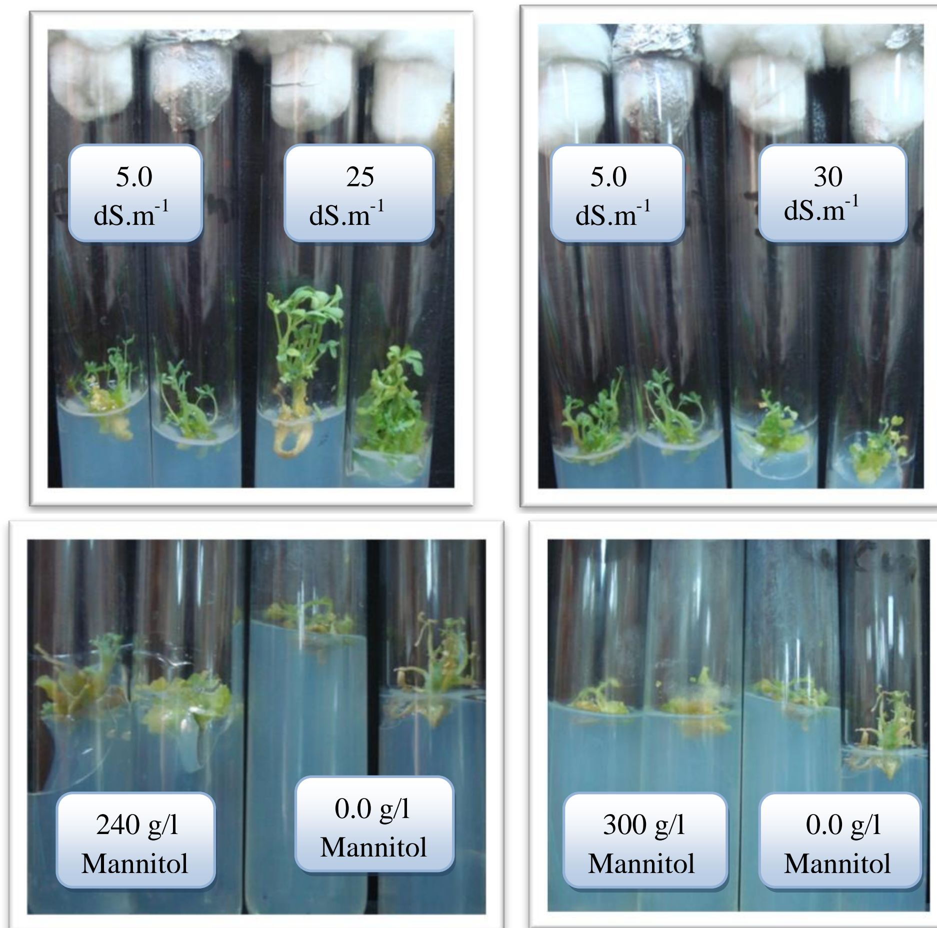


Figure (3.16): *In vitro* regeneration of *R. graveolens* callus cultures grown in MS medium supplemented with 1.5 mg/l BA and 0.5 mg/l NAA for 12 weeks, after using both direct and gradual selection methods for salinity and drought.

3.9 Determination of proline concentrations in regenerated shoots derived from callus cultures subjected to abiotic stresses.

The concentrations of porline accumulated in regenerated shoots are displayed in figure (3.17). Results revealed that accumulation of free proline is markedly increased in all stressed shoots after using both direct and gradual selection methods compared with control. The mean proline concentration increased at 240 and 300 g/l of mannitol reached 154 and 131 µM/g fwt for direct and gradual exposure respectively compared with 0.0 g/l mannitol (128 µM/g fwt).

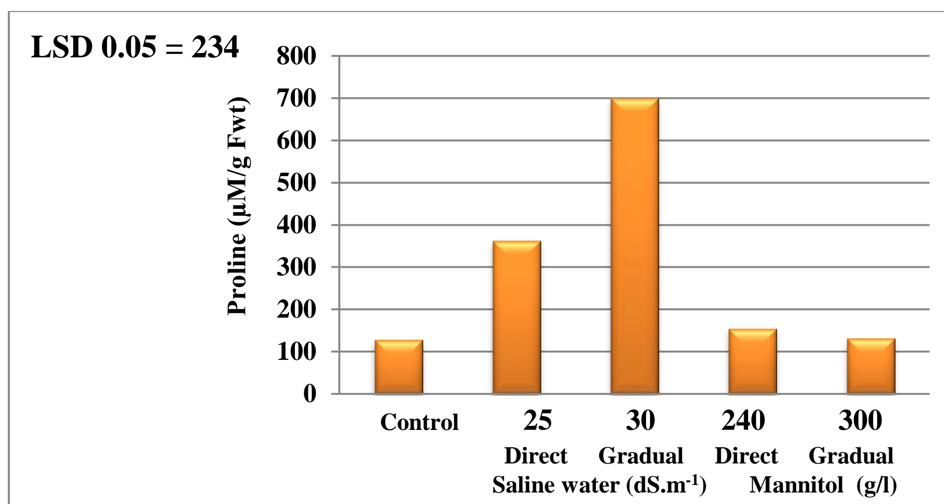


Figure (3.17): Effect of saline water ($dS.m^{-1}$) and mannitol (g/l) on the mean proline content in shoots regenerated from callus cultures subjected for salinity and drought stresses, after 12 weeks, n=3.

Direct and gradual exposure to drought stress were not significantly different from each other and with the control. These results are in line with those obtained by AL-Taha (2013) who reported that proline accumulation in shock exposure is higher than that accumulates in gradual one in sour orange callus cultures. Maximum value recorded at direct exposure to drought stress was at 240 g/l mannitol (154 µM/g fwt). Proline has long been known to accumulate in plants experiencing water limitation and both proline synthesis and catabolism are required for optimal growth at low water potential.

High concentration of proline in shoots regenerated from callus was recorded. Munns and Tester (2008) reported that the biosynthesis and accumulation of compatible solutes in plant tissues is an adaptive response of plants to drought stress and this explains the accumulation of high proline concentrations in shoots regenerated from callus cultures exposed previously to high concentrations of mannitol.

Proline concentrations in direct and gradual salinity treatments increased significantly at 25 and 30 $dS.m^{-1}$ of saline water recording 362 and 700 µM/g fwt respectively compared with a standard medium (128 µM/g fwt). Maximum amount of proline resulted in gradual exposure at 30 $dS.m^{-1}$ of saline water (700 µM/g fwt). This value is significantly different with both direct and control treatments, and they were significantly different from each other. Several studies have shown that exposure to increasing salinity concentrations caused an increase in proline content such as *Hordeum marinum* and *Hordeum vulgare* shoots (Garthwaite *et al.*, 2005); *Oryza sativa* callus (Ahmad *et al.*, 2007); *Saccharum* callus (Errabii *et al.*, 2007); *Phaseolus vulgaris* callus (Stoeva and Kaymakanova, 2008) and pepper somatic embryo (Marroquin *et al.*, 2011).

Our results are in accordance with those of Summart *et al.* (2010) and Peleg *et al.* (2011) who reported that increasing compatible solutes synthesis is a strategy to improve tolerance to abiotic stresses. Proline, is a compatible solute playing a predominant role in protecting plants from osmotic stress thus high concentrations accumulate in the cell cytoplasm without interfering with cellular structure or metabolism (Hani and Heidari, 2008; Peleg *et al.,* 2011; Rai *et al.*, 2011). It has been shown to be an effective means for enhancing plant abiotic stress tolerance (Qin *et al.*, 2011).

3.10 Determination of gallic acid concentration in regenerated shoots derived from callus cultures

The concentrations of gallic acid in plantlets were exhibited in figure (3.18). Results revealed that accumulation of gallic acid is markedly increased in all stressed plantlets in both direct and gradual selection compared with the control. The concentrations of gallic acid were almost

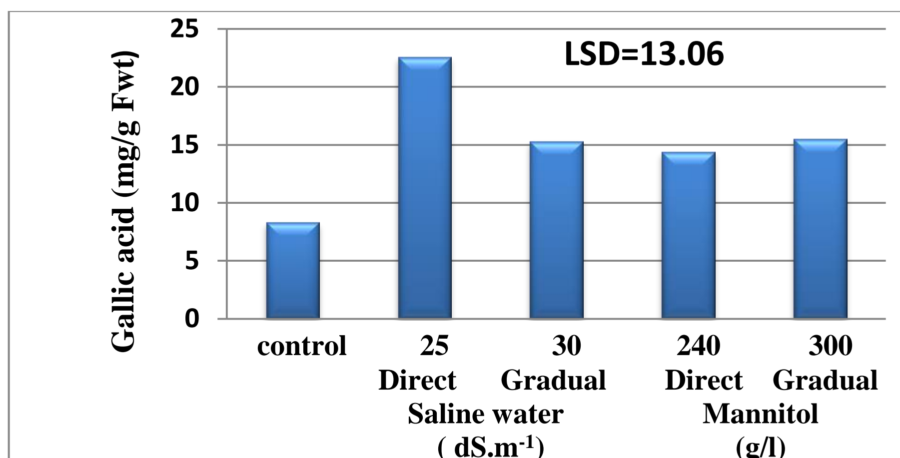


Figure (3.18): Effect of saline water (dS.m^{-1}) and mannitol (g/l) on the mean gallic acid content in *R. graveolens* shoots regenerated from callus cultures subjected to salinity and drought stresses, after 12 weeks, n=3.

similar at 30 dS.m^{-1}, 240 and 300 g/l mannitol reached 15.3, 14.4 and 15.5 mg/g fwt respectively compared with the control (8.3 mg/g fwt). These values were not significantly different with the control (appendix 2). The direct exposure to salinity stress recorded the highest value of gallic acid reached 22.6 mg/g fwt, and this is significantly different from the control. These results are in line with those reported by Taha and Hussan (2014) who found that 1.5 g/l of sea water achieved the highest content of total phenols in date palm.

Hussein and Aqlan (2011) reported that the high concentrations of mannitol enhance total phenolics and flavonoid contents in *Fenugreec* callus culture. Lechno *et al.* (1997) reported that NaCl increases the activities of the antioxidative enzymes, these activities may be directly linked to the content of phenols, tannins and flavonoids and consequently to their free radical scavenging activities.

3.11 Determination of rutin concentration in regenerated shoots derived from callus cultures

The concentrations of rutin extracted from different plantlets were exhibited in figure (3.19). Direct exposure to abiotic stresses increased

the rutin content in regenerated shoots from selected tissues (appendix 3). Rutin concentrations at 30 dS/m^{-1} saline water, 240 and 300 g/l mannitol reached 77, 145 and 51 mg/g fwt respectively compared with the control (34 mg/g fwt). The direct exposure at 25 $dS.m^{-1}$ saline water recorded the highest value reached 311 mg/g fwt.

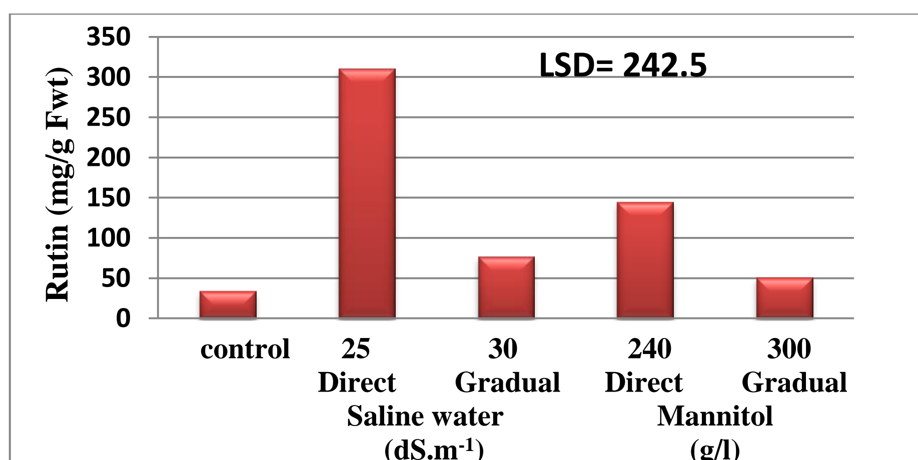


Figure (3.19): Effect of saline water ($dS.m^{-1}$) and mannitol (g/l) on the mean rutin content in *R. graveolens* shoots regenerated from callus cultures subjected to salinity and drought stresses, after 12 weeks, n=3.

This is significantly different with both control and gradual exposure to mannitol at 300 g/l only. These results are in accordance with those reported by Mohamed and Ibrahim (2012) who concluded that rutin levels are proportionally increase after raising mannitol concentrations in *Ruta* shoot culture. Garg (2010) reported that total phenols increased linearly in *Catharanthus roseus* shoots after exposure to different levels of NaCl. Pare and Tumlinson (1999) exhibited that stress induced by salinity, is associated with metabolic changes, including increased synthesis of phenolic acids that are stimulated in response to stress. While Tang and Newton (2004) stated that phenolics related positively to *in vitro* proliferation.

3.12 Determination of xanthotoxin concentration in regenerated shoots derived from callus cultures

Xanthotoxin was determined in regenerated shoots from selected callus (figure 3.20). Results exhibited that both direct exposure to salinity and gradual exposure to drought increased the concentrations of xanthotoxin in shoots (appendix 4).

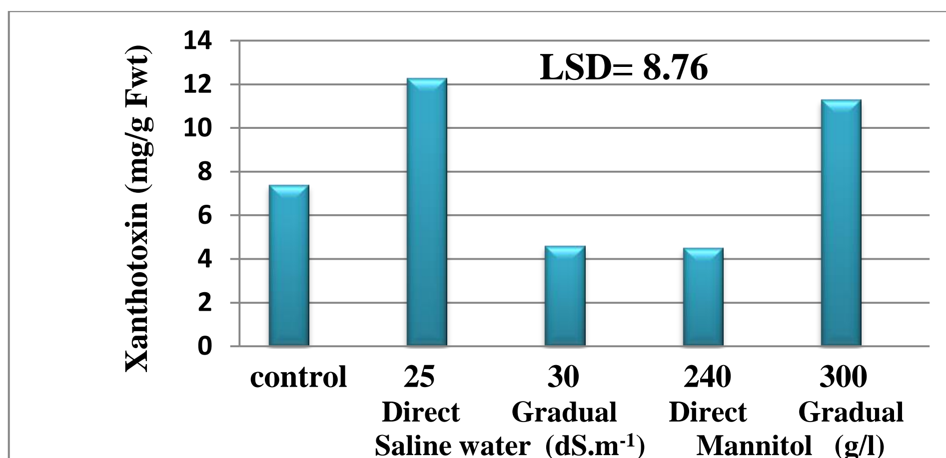


Figure (3.20): Effect of saline water ($dS.m^{-1}$) and mannitol (g/l) on the mean xanthotoxin content in *R. graveolens* shoots regenerated from callus cultures subjected to salinity and drought stresses, after 12 weeks, n=3.

Xanthotoxin level increased 25 $dS.m^{-1}$ saline water and 300 g/l mannitol reached 12.3 and 11.3 mg/g fwt respectively compared with the control (7.4 mg/g fwt). Lower xanthotoxin levels were recorded at 30 $dS.m^{-1}$ saline water and 240 g/l mannitol reached 4.6 and 4.5 mg/g fwt respectively. These results corroborate those of Mohamed and Ibrahim (2012) who revealed that xanthotoxin levels were linearly increased by raising mannitol concentrations in *Ruta* shoot cultures. Bohnert *et al.* (1995) reported that the concentrations of secondary products are strongly dependent on the growth conditions, and any type of stress has a major impact on their synthesis and accumulation. Sharma and Ramawat (2013) reported that phenolic compounds gradually elevate in NaCl treated

callus. There is no doubt that the employment of stress factors, such as osmotic shock, is an important strategy for the improvement of the secondary metabolite production in plant cell cultures (Zhao *et al.,* 2001).

3.13 Determination of bergapten concentration in regenerated shoots derived from callus cultures

The concentrations of bergapten in tissue samples taken from different regenerated shoots were exhibited in figure (3.21).

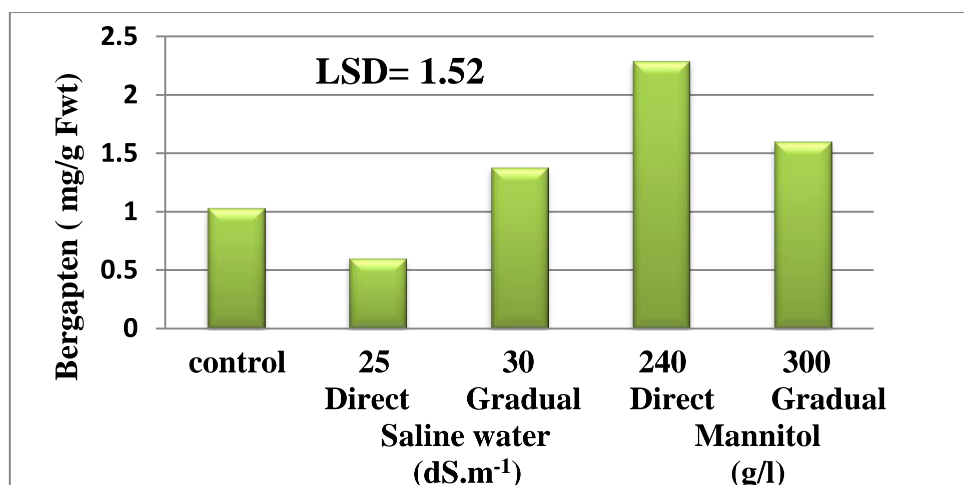


Figure (3.21): Effect of saline water ($dS.m^{-1}$) and mannitol (g/l) on the mean bergapten content in *R. graveolens* shoots regenerated from callus cultures subjected for salinity and drought tolerance, after 12 weeks, n=3.

Results indicate that bergapten concentrations increased at 30 $dS.m^{-1}$ saline water, 240 and 300 g/l mannitol reached 1.38, 2.29 and 1.6 mg/g fwt respectively compared with the control (1.03 mg/g fwt). The direct exposure to salinity at 25 $dS.m^{-1}$ recorded the lowest value for bergapten reached 0.6 mg/g fwt. The highest mean value of bergapten was recorded after direct exposure to mannitol reached 2.29 mg/g fwt (appendix 5). No significant differences between the value of both direct and gradual exposure to salinity and drought. Mohamed and Ibrahim (2012) reported that bergapten concentrations increase after raising mannitol levels in the culture medium of *Ruta* shoots.

Ayaz *et al*. (2000) found that, the increase in phenolic acids content could be related with increasing level of amino acids synthesis in cells induced during water stress. Shehab *et al.* (2010) reported high phenols accumulated in rice plantlets after exposure of callus cultures to drought stress. Hussein and Aqlan (2011) revealed that higher salt concentration significantly decreased secondary metabolites accumulation in *Trigonella foenum* callus culture. Bressan *et al.* (2002) mentioned that acidic compounds incorporating phenolic groups have been repeatedly implicated as active antioxidants.

Pan *et al.* (2006) reported that under stress treatments, some secondary metabolite concentrations increase with the progression of drought and salinity, while other compounds are decline, indicating that stress degree and the level of these compounds are affected by the degree of stress and duration time. Under stress conditions ROS levels can dramatically increase leading to oxidative stress, causing a serious imbalance between the production of ROS and antioxidant defense and this situation can cause damage to cellular macromolecules. ROS under steady-state conditions are scavenged by various antioxidant defense systems: both enzymatic antioxidant and non-enzymatic like secondary metabolites such as phenolic compounds defense systems (Sen, 2012).

3.14 Salinity and drought gain in regenerated plantlets

The plantlets regenerated from calli treated previously with 25 $dS.m^{-1}$ saline water directly, were selected and re-subjected to 25 $dS.m^{-1}$ of saline water (figure 3.22). Plantlets salt tolerance gains (%) were 0, 34, 46, 75 and 63% at the exposure times 0, 10, 20, 30 and 40 days respectively. Thus, a proportionally increase in salt tolerance with increasing the exposure time till the day 30 when untreated plantlets were died.

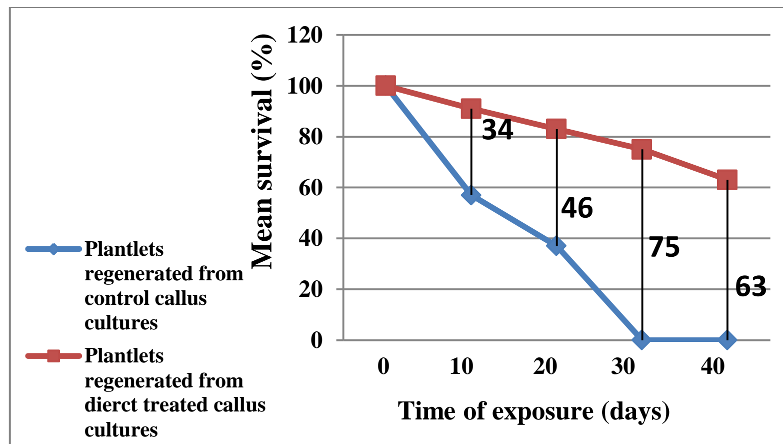


Figure (3.22): % Plantlets regenerated from directly treated and non treated callus cultures after exposure of these plantlets to 25 $dS.m^{-1}$ saline water for 40 days, lines connecting each two points represent the total gain in salt tolerance between selected and non selected plantlets.

The plantlets regenerated from calli treated previously with 30 $dS.m^{-1}$ saline water gradually, were selected and re-subjected to 30 $dS.m^{-1}$ of saline water (figure 3.23). Plantlets gain percentages were 0, 38, 50, 80 and 68% at the exposure times 0, 10, 20, 30 and 40 days respectively. Thus, a proportionally increase in salt tolerance with increasing the exposure time till the day 30 when untreated plantlets were died. These results are in line with those of Ibrahim *et al*. (1991) who reported that a remarkable gain in salt tolerance was recorded when they selected *Coleus blumei* using direct and stepwise selection techniques.

Munns and Tester (2008) reported that at the cellular level, high amounts of Na^+ and Cl^- accumulate in leaves can be tolerated by anatomical adaptations and intracellular partitioning. Yousif (2002) reported that high % survival for rice plantlets produced previously from calli treated with NaCl directly. The strategy of tolerance involves coordination of physiological and biochemical alteration at the cellular and molecular levels (Rai and Takabe, 2005; Peleg *et al*., 2011).

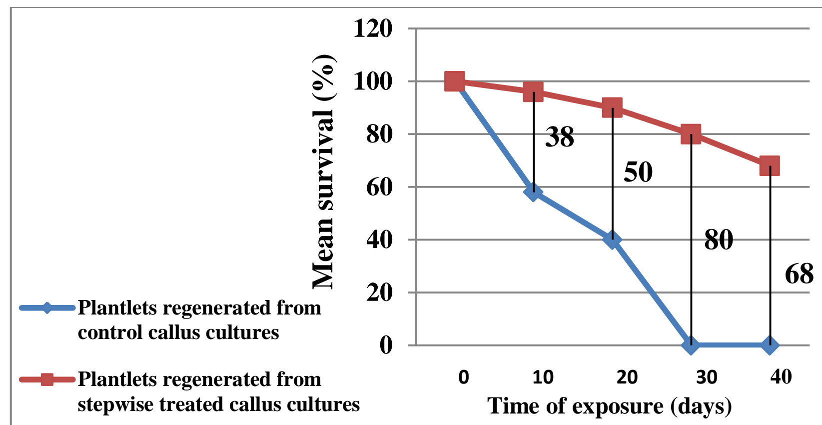


Figure (3.23): % Plantlets regenerated from stepwise treated and non treated callus cultures after exposure of these plantlets to 30 $dS.m^{-1}$ saline water for 40 days, lines connecting two each points represent the total gain in salt tolerance between selected and non selected plantlets.

The gain for salt tolerance in plantlets regenerated after direct and stepwise salt screening methods may be due to increasing the expression of genes responsible for osmoregulation particulary proline accumulation and total phenolic compounds such as rutin, xanthotoxin and gallic acid compared with control plantlets. Porline serves as an organic nitrogen reservoir ready to be used after stress relief in order to sustain both amino acid and protein synthesis. Besides, being an osmoprotectant. Proline also has a role in, stabilization of proteins and protein complexes at stress treatments and as a signaling/regulatory molecule (Hong *et al*., 2000; Mademba *et al*., 2003 and Lokhande and Suprasanna, 2012).

The plantlets regenerated from calli treated previously with 240 g/l mannitol directly, were selected and re-subjected to 240 g/l of mannitol (figure 3.24). Plantlets drought tolerance gains (%) were 0, 45, 50, 70 and 61% at the exposure times 0, 10, 20, 30 and 40 days respectively. Drought tolerance increased with increasing the exposure time till the day 30 when control plantlets were terminated. The plantlets regenerated from

calli treated previously with 300 g/l mannitol gradually, were selected and re-subjected to 300 g/l mannitol (figure 3.25). Plantlets survival percentages were 0, 44, 53, 72 and 63% at the exposure times 0, 10, 20, 30 and 40 days respectively. The trend of increase and reduction was similar to that of direct method. Gain value was dropped to 63% after exposure of these plantlets to mannitol after 40 days.

The osmotic stress affected the growth of shoots clearly after exposure to 300 g/l mannitol for control plantlets. A desiccation appeared on young leaves. These results are in line with those reported by Placide *et al.* (2012) who reported that exposure of banana plantlets to drought stress reduced the leaves growth.

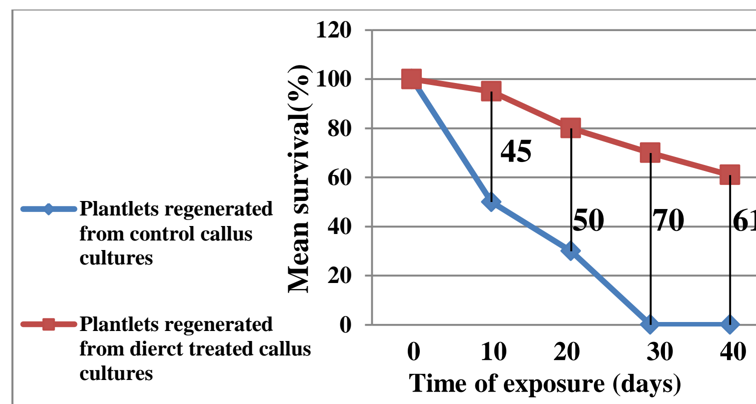


Figure (3.24): % Plantlets regenerated from directly treated and non treated callus cultures after exposure of these plantlets to 240 g/l mannitol for 40 days, lines connecting each two points represent the total gain in drought tolerance between selected and non selected plantlets.

Drought conditions decrease the plant metabolic pathways, the amount of metabolites and plant biomass (Kulkarni and Phalke, 2009). Plants alter metabolism in various ways to accumulate abiotic stresses including producing osmoregulatory compounds such as proline (Perez and Gomes, 2012). The complex plant response to abiotic stress involves many genes and biochemical-molecular mechanisms.

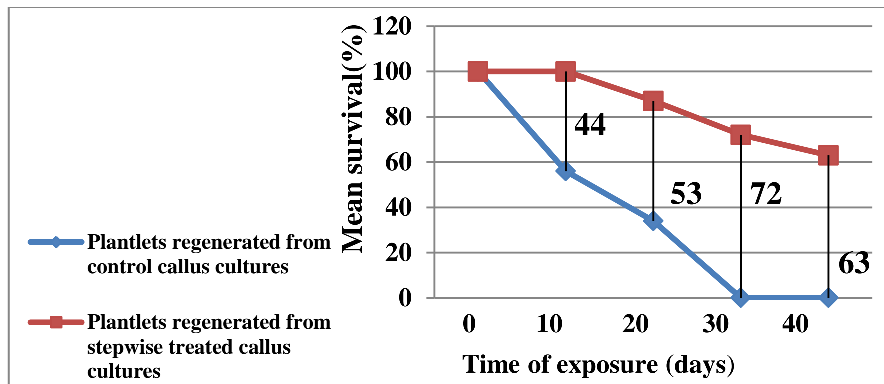


Figure (3.25): % Plantlets regenerated from stepwise treated and non treated callus cultures after exposure of these plantlets to 300 g/l mannitol for 40 days, lines connecting each two points represent the total gain in drought tolerance between selected and non selected plantlets.

Various genes respond to drought-stress by increasing the expression of these genes (Rodrigues *et al.,* 2005). The gain for drought tolerance in plantlets from direct and stepwise drought screening methods may be due to increasing the expression of genes responsible for xanthotoxin, bergapten and gallic acid accumulation. Identified the genes associated with drought tolerance.

3.15 Differential display reverse transcription polymerase chain reaction (DDRT-PCR)

polymorphisms at cDNAs levels in drought and salinity tolerant *R. graveolens* regenerated plantlets were compared with control cDNA (regenerated plants not subjected to stress) and DNA from intact plants. cDNA was amplified with ten base arbitrary primers. The polymorphic fragments capable of differentiating the tolerant lines from the stressed regenerated plantlets were generated by most primers. New bands were observed in salinity and drought stressed regenerated plantlets which were not detected in cDNA obtained from control.

The marker OPA-01 generated a profile distinguished the tolerant lines in stress regenerated plantlets from the control plantlets (figure

3.26). The intensity of the bands increased in the regenerated stressed plantlets. Amplification with the primer revealed the presence of a 646 bp fragments in tolerant lines (lanes 3, 4, 5 and 6) compared with the control. Onthe other hand, fragments at 676 pb in DNA from intact plant (lane 1) and at 688 and 1014 bp in cDNA from control plantlet (lane 2) has not been detected in tolerant lines. A new bands with a molecular size of about 770 bp were observed in plantlets produced from direct salt screening method (lane 3) and plantlets produced from direct and stepwise drought screening method (lanes 5 and 6).

Band at a size of 955 bp was detected in plantlets produced from direct salt screening method (lane 3) and plantlets produced from direct drought screening method (lane 5), while a band at 983 bp was detected in plantlet produced from stepwise drought screening method (lane 6).

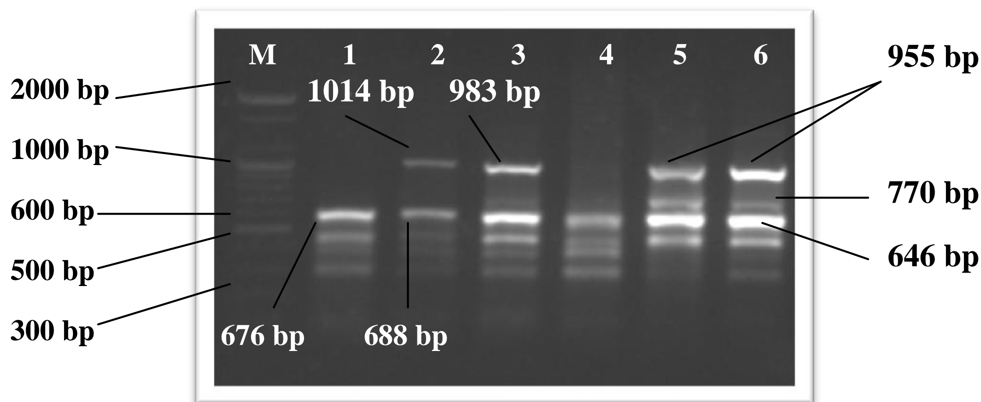


Figure (3.26): Amplification profile of differential display obtained using the primer OPA-01, lane (1): DNA from intact plant, lane (2): cDNA from regenerated non stressed plantlet, lanes (3,4): cDNA from regenerated plantlets subjected to direct or gradual exposure to saline water, lanes 5,6,7: cDNA from regenerated plantlets subjected to direct or gradual exposure to mannitol, M: ladder 200 bp.

However these bands were not found in plantlets produced from control treatment (lanes 1 and 2). The marker OPA-08 amplified seven bands, of which five were polymorphic, two were monomorphic. The primer has an amplification product with molecular sizes about 988 and 593 bp fragments visualized in all tolerant plantlets (lanes 3, 4, 5 and 6) and in DNA from intact plant (lane 1) , but it was absent in cDNA from control plantlet (lane 2) figure (3.27).

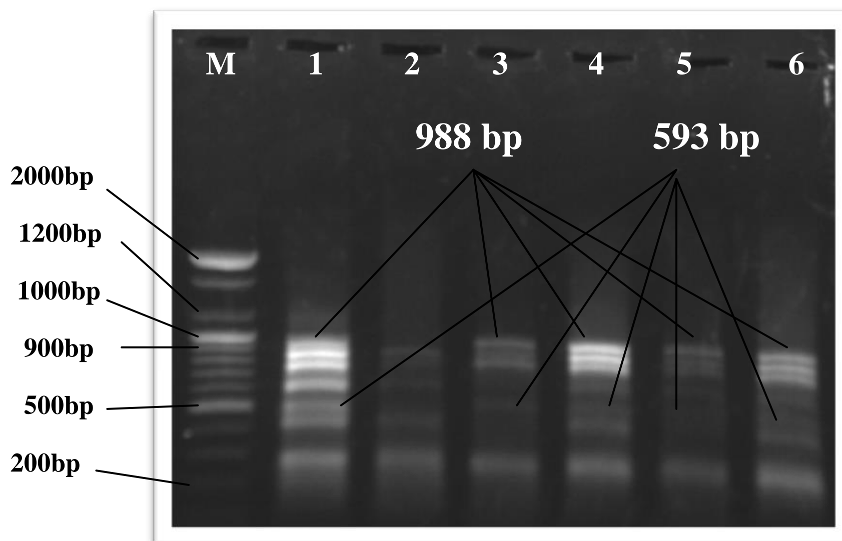


Figure (3.27): Amplification profile of differential display obtained using the primer OPA-08, lane (1): DNA from intact plant, lane (2): cDNA from regenerated non stressed plantlet, lanes (3,4): cDNA from regenerated plantlets subjected to direct or gradual exposure to saline water, lanes (5,6): cDNA from regenerated plantlets subjected to direct or gradual mannitol, M: ladder 200 bp.

Amplification products using the primer OPA-11 (figure 3.28 a) shows the presence of a new bands with molecular sizes 831and 674 bp in the lanes 3, 4, 5, 6, and in lane 1 of the control but they are missing in lane 2 of the control. The primer amplified band with a molecular size 719 bp showed high intensity as visualized in lanes 3,4,5, 6 and 1 but it exhibited very low intensity in lane 2. While two bands with a molecular

size 541 and 300 bp was visualized in the all tolerant lines but not found in control plantlets (lane 1 and 2).

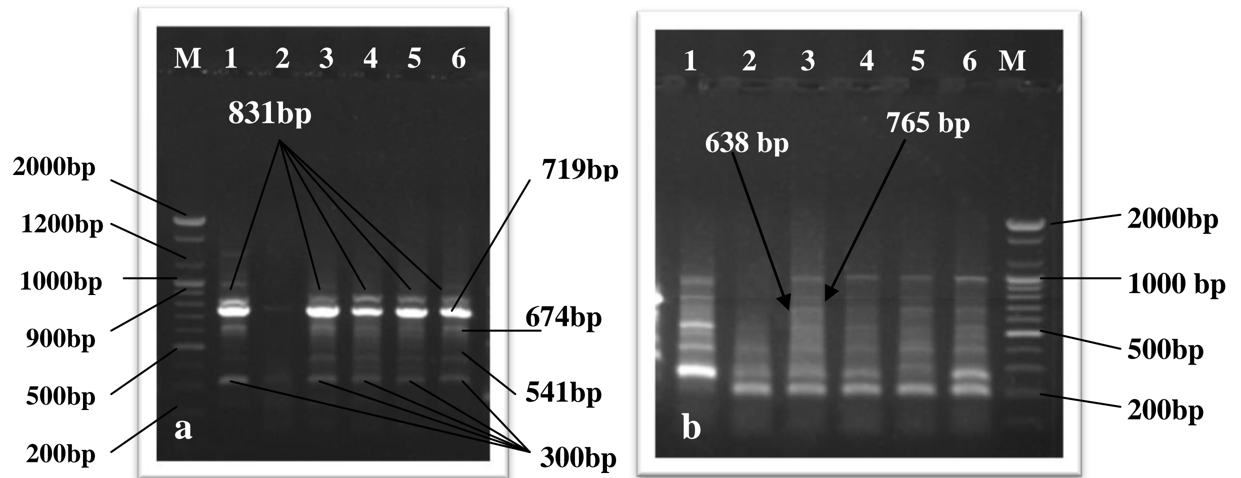


Figure (3.28): Amplification profile of differential display obtained using the primer OPA-11 (a) and OPA-17 (b), lane (1): DNA from intact plant, lane (2): cDNA from regenerated non stressed plantlet, lanes (3,4): cDNA from regenerated plantlets subjected to direct or gradual exposure to saline water, lanes (5,6): cDNA from regenerated plantlets subjected to direct or gradual exposure to mannitol, M: ladder 200bp.

Amplification with the marker OPA-17 revealed the presence of a 1000, 765 and 638 bp fragments in all tolerant plantlets in lanes 3,4,5 and 6 compared with the control in the lane 1 but those were absent in the cDNA of the control regenerated plantlets in lane 2 (figure 3.28 b).

The primer OPA-15 amplified three monomorphic bands were detected in all samples (figure 3.29 a). One band with a molecular size 900 bp as visualized in plantlets produced from direct salt screening method (lane3) and plantlets produced from direct and stepwise drought screening method (lane 5 and 6) in the control plant (lane 1) but this band was absent in plantlet from stepwise salt screening method (lane 4) and in the cDNA of the control (lane 2). While another band was detected in lane 3 only with a molecular size 645 bp.

Amplification with the marker OPB-05 (figure 3.29 b) shows the presence of a band at size 290 bp only in plantlets from stepwise salt and direct drought screening methods (lanes 4 and 5). While a band with the molecular size 1013 bp was visualized only in plantlet produced from stepwise drought screening method (lane 6).

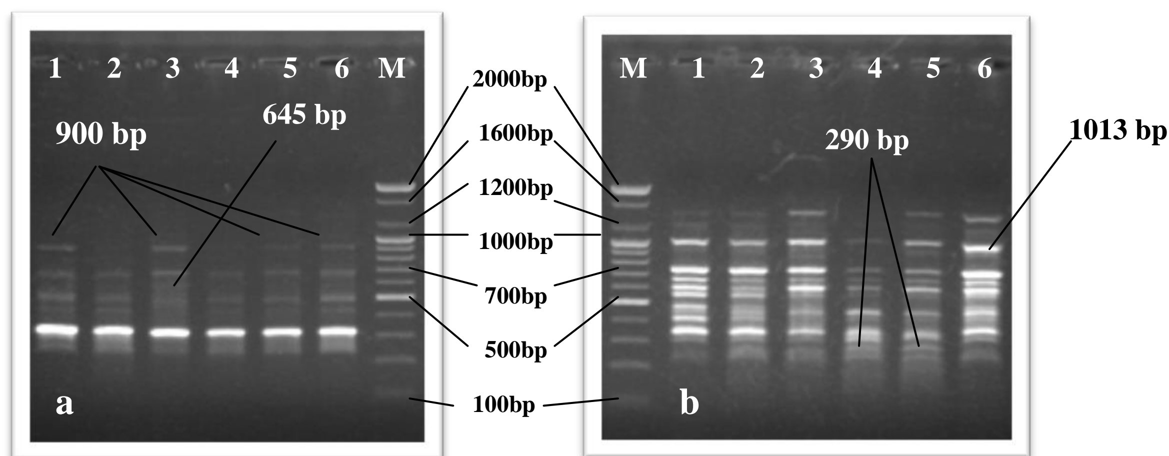


Figure (3.29): Amplification profile of differential display obtained using the primer OPA-15 (a) and OPB-05 (b), lane (1): DNA from intact plant, lane (2): cDNA from regenerated non stressed plantlet, lanes (3,4): cDNA from regenerated plantlets subjected to direct or gradual exposure to saline water, lanes (5,6): cDNA from regenerated plantlets subjected to direct or gradual exposure to mannitol, M: ladder 200 bp.

Many bands that amplified by the marker OPA-05 with molecular sizes 100-300 bp were appeared in all tolerant plantlets but were missing in the control ones (figure 3.30 a). The intensity in the band at 870 bp appeared clearly in all tolerant plantlets while lower intensity band exhibited in control plantlets (lanes 1 and 2).

The marker OPA-10 generated a profile differentiating the tolerant plantlets from the control ones (figure 3.30 b). Amplification products with this primer illustrated the presence of 1000 and 500 bp visualized in salt tolerant plantlets (lanes 3,4) and in those produced from stepwise

drought screening method (lane 6), while 255 bp was clear in lanes 4 and 6 only.

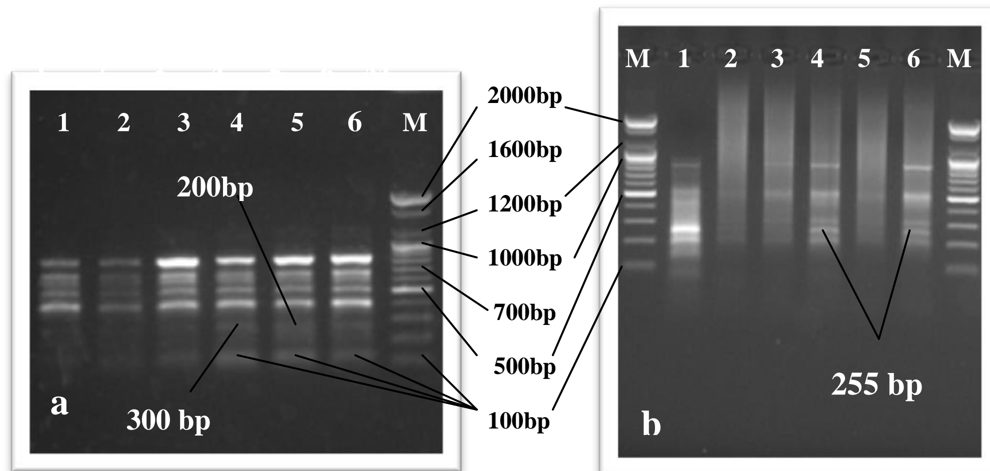


Figure (3.30): Amplification profile of differential display obtained using the primer OPA-05 (a) and OPA-10 (b), lane (1): DNA from intact plant, lane (2): cDNA from regenerated non stressed plantlet, lanes (3,4): cDNA from regenerated plantlets subjected to direct or gradual exposure to saline water, lanes (5,6): cDNA from regenerated plantlets subjected to direct or gradual exposure to mannitol, M: ladder 200 bp.

These bands were absent in the control plants. The two primers OPC-04 and OPE-08 showed many amplification products in all tested samples however, no new bands appeared in the regenerated tolerant plantlets (figure 3.31 a and b).

DNA fingerprinting by DDRT-PCR with RAPDs confirmed that the tolerant regenerates differed genotypically from the control plantlets and also revealed genetic polymorphism between the selected salt and drought lines. At annealing in PCR conditions, even slight base change at the primer-annealing site can result in esence or absence of RAPD bands. It is therefore concluded that somaclonal variation under tissue culture conditions regenerated genetic changes among the selected plantlets.

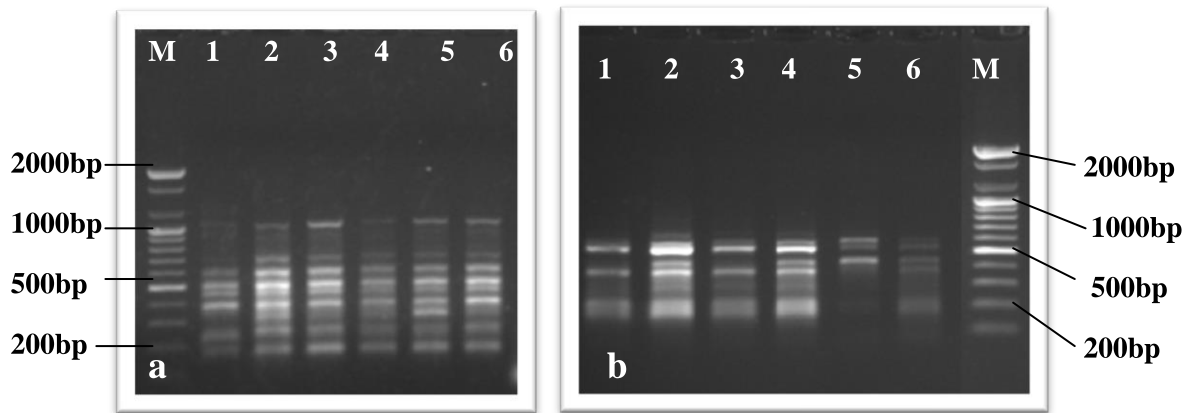


Figure (3.31): Amplification profile of differential display obtained using the primer OPC-04 (a) and OPE-08 (b), lane (1): DNA from intact plant, lane (2): cDNA from regenerated non stressed plantlet, lanes (3,4): cDNA from regenerated plantlets subjected to direct or gradual exposure to saline water, lanes (5,6): cDNA from regenerated plantlets subjected to direct or gradual exposure to mannitol, M: ladder 200 bp.

somaclonal variations play an important role in varietal improvement. It is proven that some tissue culture variants are superior than the donor clones in terms of biotic and abiotic resistance (Balkrishna and Shankarrao, 2013; Patade *et al*., 2006; Rajeswari *et al*., 2009
; Shomeli *et al*., 2011). Cellular and molecular mechanisms behind the somaclonal variations are mitotic irregularities leading to chromosomal instability, occurrence of gene amplification or deletion, gene inactivation or reactivation of silent genes, transposition and somatic crossing over (Larkin and Scowcroft 1981; Muller *et al.,* 1990). Activation of complex signaling pathway (s) bring about drastic changes in the cellular gene expression which is a perquisite for plants to acclimatize under extreme conditions (Tong *et al*., 2007; Tamirisa *et al.,* 2014).

DDRT-PCR has been widely used to identify and analyze the expression patterns of previously uncharacterized genes in many different species. Since this technique is very efficient, a large number of

differentially expressed genes can usually be identified specially those expressed under stressful conditions in plants (Alves *et al.,* 1998). In the present study, the total amplification products generated by these primers ranged from 200 bp in primer OPC-04 (figure 3.31 a) to approximately 1400 bp in primer OPB-05 (figure 3.29 b), and the result confirm that cDNAs are differentially express in response to either drought or salinity stresses. Abiotic stress conditions such as drought and salinity cause adverse effects on the overall plant growth.

Salinity and drought stresses are known to activate certain sets of common genes in a plant cell. Many responsive genes that express under stress conditions are classified into two groups; the first includes different classes of proteins such as enzymes required for biosynthesis of various osmoprotectants, LEA proteins, chaperones, and detoxification enzymes, which directly protect cells. While the second group includes signaling molecules, transcription factors, and protein kinases (Rai *et al*., 2011; Lokhande and Suprasanna, 2012).

Mahajan and Tuteja (2005) reported that each stress is under multigenic control and therefore its manipulation may result in alteration of a large number of genes as well as their products. Thus, this may explain the differences in number and intensity of cDNAs bands at different concentrations of salinity and drought exposure compared with those bands visualized in the of control plantlets. Tamirisa *et al.* (2014) proposed an evidence that enhanced abiotic stress tolerance of Arabidopsis transgenic plants is due to a significant increase in the expression levels of different abiotic stress responsive genes.

Kamal *et al*. (2010) reported that proteins function expressed by genes in stress tolerant and susceptible plants will not only advance plant adaptation but also enhance tolerance to environmental stresses. The findings of the present study indicated that the DDRT-PCR technique is

suitable for determining the expression of defense-related genes in tolerant regenerated plantlets of *R. graveolens*.

Conclusions

According to this study, the following conclusions are recommended:

1. Callus cultures were induced on MS medium supplemented with 0.5 mg/l Kin and 1.5 mg/l 2,4-D for the leaf explants while for stem explants was 1 mg/l Kin and 1mg/l 2,4-D, then callus maintained on MS medium supplemented with 0.5 mg/l Kin and 1 mg/l 2,4-D. Shoots were regenerated on MS medium supplemented with 0.5 mg/l NAA and 1.5 mg/l BA, then elongated on MS medium with 0.5 mg/l GA_3.
2. Saline water and mannitol caused an increase in callus growth, shoot regeneration and proline accumulation when direct or gradual exposure to salinity and drought were imposed to select salinity and drought tolerant cell lines, the regeneration of whole plants
3. Saline water and mannitol increased the phenolic compounds, gallic acid and rutin concentrations in both direct and stepwise methods by 2.7, 9.0 folds respectively.
4. Saline water and mannitol increased furanocumarens folds in regenerated shoots at 25 $dS.m^{-1}$ saline water and 1.6 fold at 300 g/l mannitol for xanthotoxine, while bergapten concentration increased 2.3 fold at 240 g/l mannitol.
5. The gain in salt tolerance in plantlets regenerated from calli treated previously with 25 $dS.m^{-1}$ saline water directly and 30 $dS.m^{-1}$ saline water gradually increased up to 63% and 68% respectively after re-subjection to 25 and 30 $dS.m^{-1}$ saline water after 40 days respectively.
6. The gain in drought tolerance of plantlets regenerated from calli treated previously with 240 g/l mannitol directly and 300 g/l mannitol gradually increased upto 61% and 63% respectively after

re-subjected to 240 g/l mannitol and 300 g/l mannitol after 40 days respectively.

7. DDRT-PCR results confirmed that a new bands with different molecular sizes were observed in the primers OPA-01, OPA-08, OPA-11, OPA-17 and OPA-15 indicating the expression of a new genes amplified under stress conditions or may due to already existing genes.

Recommendations

According to this study, we suggested the following recommendations:

1. Using the tolerant *R. graveolens* plants produced in this study as ground cover in lands exposed to salinity and drought.
2. Evaluating the ability of *R. graveolens* to tolerate other stresses especially heavy metals.
3. Investigating the type and sequence of genes expressing salinity and drought in cDNA using DNA sequencer.
4. Using genetic engineering techniques for transferring genes from salt and drought tolerant plants to sensitive ones particularly within compatible species.

References

References

- Aazami, M. A.; Torabi, M. and Shekari, F. (2010). Response of some tomato cultivars to sodium chloride stress under *in vitro* culture condition. Afric. J. Agric. Res., 5(18): 2589-2592.
- Abbas, M. F.; Jasim, A. M. and AL-Zubaidy, B. H. (2012). The effect of proline on growth and ionic composition of embryogenic callus and somatic embryos of the date palm (*Phoenix dactylifera* L. cv. Ashkar) under NaCl stress. Int. J. Farm. Allied Sci., 1(3): 82-87.
- Abdel – Raheem, A. T.; Ragab, A. R.; Kasem, Z. A. and Samera, A. M. (2007). *In vitro* selection for tomato plants for drought tolerance via callus culture under polyethylene glycol (PEG) and mannitol treatments. Afric. Crop Sci. Confer. Proceed., 8: 2027-2032.
- Abdel Rahman, R.; Gomaa, S. E.; Abdelsalam, N. R.; EL-Wakil, H.; Khaledi, A. and Hassan, H. M. (2013). Effect of sodium chloride on tropane alkaloids accumulation and proline content in *Datura metel* and *D. stramonium* callus cultures. Int. J. Adv. Biol. Biomed. Res., 1(2): 197-210.
- Abdi, G.; Hedayat, M. and Khush-Khui, M. (2011). Development of NaCl-tolerant line in *Tanacetum cinerariaefolium* (Trevin) Schultz-Bip through shoot organogenesis of selected callus line. J. Biol. Environ. Sci., 5(15): 111-119.
- Abdul-Baker, B. R. (2012). Study on microprpagation of two varieties of tomato (*Lycopersicon esculentum* Mill.) and their response to water stress by PEG. MSc Thesis, College of Education, University of Basrah, Basrah, Iraq.

- Abdul-Jaleel, C. (2009). Changes in non-enzymatic anti oxidants and ajmalicine production in *Catharanthus roseus* with different soil salinity regimes. Botany Rev. Int., 2(1): 1-6.
- Abdul-Jaleel, C.; Sankar, B.; Sridharan, R. and Panneerselvam, R. (2008). Soil salinity alters growth, chlorophyll content, and secondary metabolite accumulation in *Catharanthus roseus*. Turk. J. Biol., 32: 79-83.
- Addae Frimpomaah, F. A.; Arkorful, E. and Tengey, T. K. (2014). The effect of 2,4-D on callus induction using leaf lobe of sweet potato as a source of explants. Int. J. of Agronomy and Agric. Res., 5(1): 16-22.
- Ahmad, A.; Faisal, M.; Anis. M. and Aref. I. M. (2010). *In vitro* callus induction and plant regeneration from leaf explants of *Ruta graveolens* L. South Afric. J. Botany, 76: 597-600.
- Ahmad, M. S. A.; Javed, F. and Ashraf, M. (2007). Iso-osmotic effect of NaCl and PEG on growth, cations and free proline accumulation in callus tissue of two indica rice (*Oryza sativa* L.) genotypes. Plant Growth Regul., 53: 53-63.
- Al-Bahrany, A. M. (2002). Callus growth and proline accumulation in response to polyethylene glycol induced osmotic stress in rice *Oryza sativ*a L. Pak. J. Biol. Sci., 5(12): 1294–1296.
- AL-Kazaz, A. A. (2001). Isolation and characterization of salt-induced transcript from halophyte *Spartina anglica* using a modified version of DDRT-PCR. PhD thesis, College of Science, Nankai University, China.
- AL-Taee, K. N. K. and AL-Humairi, B. A. J. (2013). Treatment of water drainage for agriculture. Global J. of Res. Eng., 13(6-E): 50-59.

- AL-Taha, H. A. K. (2013). Effect of shock and gradual drought by PEG on callus growth and proline accumulation in sour orange *(Citrus x aurantium*). Adv. in Agric. and Bot. Int. J. of Biol. Soc., 5(2): 77-83.
- Alves, J. D.; VanToai, T. and Kaya, N. (1998). Differential display: A novel PCR – based method for gene isolation and cloning. Revista Brasileria de Fisiologia Vegetal., 10(2): 161-164.
- Amudha, J. and Balasubramani, G. (2011). Recent molecular advances to combat abiotic stress tolerance in crop plants. Biotech. and Mol. Biol. Rev., 6(2): 31-58.
- Antoniw, J. F.; Ritter, C. E.; Pierpoint, W. S. and Van, L. C. (1980). Comparison of three pathogenesis-related proteins from plants of two cultivars of tabocco infected with TMV. J. Gen. Virol., 47: 79-87.
- Arivalagan, U.; Alderson, P. G. and Nagarajan, A. (2012). Effect of growth hormones on callus induction of *Sauropus androgynous* (sweet shoot). Anal. Biol. Res., 3(10): 4668-4674.
- Asilbekova, D. T.; Gusakova, A. I.; Glushenkova, A. R., Azizkhodzhaev, A.; Erkkenova, E. M. and Sakhibaeva, M. (1993). Lipids of the biomass of *Ruta graveolens,* grown *in vivo* and *in vitro*. Chem. Natr. Compounds, 29(5): 574-577.
- Ayaz, F. A.; Kadioglu, A. and Turgut. R. (2000). Water stress effects on the content of low molecular weight carbohydrates and phenolic acids in *Ctenanthe setosa*. (Rosc.) Eichler. Can. J. Plant Sci., 80: 373-378.
- Azhar, N.; Hussain, B.; Ashraf, M. Y. and Abbasi, K. Y. (2011). Water stress mediated changes in growth, physiology and

secondary metabolites of desi ajwain (*Trachyspermum ammi* L.). Pak. J. Bot., 43: 15-19.

- Balal, R. M.; Ashraf, M. Y.; Khan, M. M.; Jaskani, M. J. and Ashfaq, M. (2011). Influence of salt stress on growth and biochemical parameters of citrus rootstocks. Pak. J. Bot., 43(4): 2135-2141.
- Balkrishna, R. and Shankarrao, S. (2013). *In vitro* screening and molecular genetic markers associated with salt tolerance in maize. Afr. J. Biotech., 12(27): 4251-4255.
- Barceloux, D. G. (2008). Rue (*Ruta graveolens* L.). In:Medical Toxicology of Natural Substances: Foods, Fungi, Medicinal Herbs, Plants, and Venomous Animals. NJ: Willy & Sons. pp: 579-581.
- Batanouny, K. (2012). A Guide to Medicinal Plants in North Africa Centre for Mediterranean Cooperation, International Union for Conservation of Nature and Natural Resources: *Ruta chalepensis* L. pp: 241-244.
- Bates, L. S., Waldren, R. and Teare, I. D. (1973). Rapid determination of free proline for water -stress studies. Plant and Soil, 39: 205-207
- Baumert, A.; Groger, D.; Kuzoukina, I. and Reisch, J. (1992). Secondary metabolites produced by cell culture of various *Ruta*. Plant Cell Tiss. Org. Cult., 28: 159-162.
- Bekheet, S. A.; Taha, H. S. and Solliman, M. E. (2006). Salt tolerance in tissue cultures of onion (*Allium cepa* L.). Arab J. Biotech., 9(3): 467-476.
- Benazir, J. F.; Suganthi, R.; Renjini Devi, M. R.; Suganya, K., Suganya, K.; Nizar, A. K. and Santhi, R. (2011). Phytochemical

profiling, antimicrobial and cytotoxicity studies of methanolic extracts from *Ruta graveolens.* J. Pharm. Res., 4(5):1407-1409.

- Berenbaum, M. R.; Nitao, J. K. and Zangerl, A. R. (1991). Adaptive significance of furnocuomarins diversity *in Pastinaca sativa* (Apiaceae). J. Chem. Ecol., 17(1): 207-215.
- Bohidar, S.; Thirunavoukkarasu, M. and Rao, T. (2008). Effect of plant growth regulators on *in vitro* microprpagation of 'garden rue' (*Ruta graveolens* L.). Int. J. of Integrative Biol., 3(1): 36-43.
- Bohlmann, J. and Eilert, U. (1994). Elicitor induced secondary metabolism in *Ruta graveolens*. Plant Cell Tiss. Org. Cult., 38: 189-198.
- Bohnert, H. J. and Cushman, J. C. (2002). Plants and environmental stress adaptation strategies. In: Plant Biotechnology and Transgenic Plants. Oksman-Caldentey, K. and Barz, W. H.(eds.). CRC press. eBook ISBN: 978-0-203-91084-9
- Bohnert, H. J.; Nelson, D. E. and Jensen, R. G. (1995). Adaptations to environmental stresses. The Plant Cell, 7: 1099–1111.
- Borges, A.; Rosa, M.; Recchia, G.; de Queiroz- Silva, J.; Bressan, E. and Veasey, E. (2009). CTAB methods for DNA extraction of sweet potato for microsatellite analysis. Sci. Agric. (Piracicaba, Braz.), 66: 529–534.
- Bouiamrine, H. and Diouri, M. (2012). Response of durum wheat (*Triticum durum* Desf.) callus culture to osmotic- induced drought stress caused by polyethylene glycol (PEG). Annal. Biol. Res., 3(9): 4555-4563.
- Bressan, R.; Hasegawa, P. and Locy, R. (2002). Stress physiology. In: plant physiology, 3rd edited. Taiz, L, and Zeiger, E. (eds.) Sinauer Associates Inc., Sunderland, Massachusetts. pp 591-623.

- Carillo, P.; Annunziata, M. G. ; Pontecorvo, G.; Fuggi, A. and Woodrow, P. (2011). Salinity stress and salt tolerance. In: Abiotic Stress in Plants - Mechanisms and Adaptations. Shanker, A. K. and Venkateswarlu, B. (eds.). InTech Publisher, Rijeka, Croatia. pp:21-39.
- Cazares, B. X.; Ortega, F. A. R.; Elenes, L. F. and Medrano, R. R. (2010). Drought tolerance in crop plants. Americ. J. of Plant Physiol., 5(5): 241-256.
- Chatzissavvidis, C.; Antonopoulou, C.; Therios, I. and Dimassi, K. (2014). Responses of trifoliate orange (*Poncirus trifoliata* (L.) Raf) to continuously and gradually increasing NaCl concentration. Acta Bot. Croat., 73 (1): 275–280.
- Chaves, M. M. and Oliveira, M. M. (2004). Mechanisms underlying plant resilience to water deficits: prospects for water-saving agriculture. J. Exp. Bot., 55: 2365-2384.
- Chen, Y.; Zahavi, E.; Barak, P. and Umiel, N. (1980). Effects of salinity stress on tobacco: 1. The growth of *N. tabacum* callus cultures under sea water, NaCl and mannitol stresses. Zeitschrift fur Pflanzephsiol., 98: 141-153.
- Chevallier, A. (1996). The Encyclopedia of Medicinal Plants: A Practical Referece Guide to over 550 Key Herbs and Their Medicinal Uses. New York, NY: DK Publishing. pp: 262-263.
- Chinnusamy, V.; Jagendorf, A. and Zhu, J. (2005). Understanding and improving salt tolerance in plants. Crop Sci., 45: 437-448.
- Cicek, N. and Cakirlar, H. (2002). The effect of salinity on some physiological parameters in two maize cultivars. Bulgar. J. Plant Physiol., 28(1-2): 66-74.

- Colgecen, H.; Koca, U. and Toker, G. (2011). Influence of different sterilization methods on callus initiation and production of pigmented callus in *Arnebia densiflora* ledeb. Turk. J. Biol., 35: 513-520.
- Couto, M. C.; Sudre, A. P.; Lima, M. F. and Bromfim, T. C. (2013). Comparison of techniques for DNA extraction and agarose gel staining of DNA fragments using samples of *Cryptosporidium*. Vet. Med., 58(10): 535-542.
- Cramer, G. R.; Urano, K.; Delort, S.; Pezzotti, M. and Shinozak, K. (2011). Effect of abiotic stress on plants: a systems biology perspective, BMC Plant Biol., 11 (1):163.
- Darvishi, H. H. and Farahani, H. A. (2010). The use of saline water for irrigation in medicinal and aromatic plants farming. Int. J. Water Res. Environ. Eng., 2(1): 9-15.
- de Almeida, D.; Santos, R. and Ribeiro-Paes, J. (2012). Exequibility of differential gene expression analysis by DDRT-PCR in murine bone marrow cells. Medicina (Ribeirão Preto), 45(4): 424-431.
- de Carvalho, M. H. C. (2008). Drought stress and reactive oxygen species. Plant Signal. Behav., 3(3): 156-165.
- de Oliveira, T. M.; Ciddade, L. C.; Gesteira, A. S.; Filho , M.; Filho, W. and Costa, M. G. C. (2011). Analysis of the NAC transcription factor gene family in citrus reveals a novel member involved in multiple abiotic stress responses. Tree Genet. Genomes, 7(6), 1123-1134.
- Dinar, M. (2009). Cost effective use of saline water for irrigation. Greenhouse. Greenhouse Articles. Saline Water for Irrigation. pp: 308.

- Diwan, R. and Malpathak, N. (2008). Novel technique for scaling up of micropropagated *Ruta graveolens* shoots using liquid culture systems: A step towards commercialization. New Biotechnol., 25(1): 85-91.
- Diwan, R. and Malpathak, N. (2010). Histochemical localization in *Ruta graveolens* cell culture: elucidating the relationship between cellular differentiation and furanocoumarin production. *In Vitro* Cell. Dev. Biology- Plant., 46: 108-116.
- Diwan, R. and Malpathak, N. (2011a). Bioprocess optimization of furanocoumarin elicitation by medium renewal and re – elicitation: a perfusion – based approach. Appl. Biochem. Biotech., 163 (6):756-764.
- Diwan, R. and Malpathak, N. (2011b). *Ruta graveolens* cultures as screening resources for phyto-pharmaceuticals: Bio-prospecting, metabolic phenotyping and multivariate analysis. Bioremed. Biodivers. and Bioavailabil., 5(1): 1-9.
- Diwan, R.; Shinde, A. and Malpathak, N. (2012). Phytochemical composition and antioxidant potential of *Ruta graveolens* L. *in vitro* culture lines. J. Botany, 2(7): 1-6.
- Dodds, J. H. and Roberts, L.W. (1985). Experiments in Plant Tissue Culture. 2edition. Press Syndicate of Cambridge University Press, Cambridage.
- Dogan, M.; Tipirdamaz, R. and Demir, Y. (2010). Effective salt criteria in callus -cultured tomato genotypes. Z Naturforsch C., 65 (9-10): 613-618.
- Dubey, R. (1994). Protein synthesis by plants under stressful conditions. In: Handbook of Plant and Crop Stress, Second

Edition. Mohammed, P. (ed.). Marcel, Dekker, New York. pp: 277-299.

- El - Sherbeny, S. E.; Hussein, M. S. and Khalil, M. Y. (2007). Improving the production of *Ruta graveolens* L. plants cultivated under different compost levels and various sowing distances. J. Agric. Environ. Sci., 2: 271-281.
- EL-Yacoubi, H.; Ayolie, K. and Rochdi, A. (2010). *In vitro* cellular salt tolerance of *Troyer citrange* : Changes in growth and solutes accumulation in callus tissue. Int. J. Agric. Biol., 12(2): 187-193.
- EL-Houssine, B. and Diouri, M, D. (2012). Response of durum wheat (*Triticum durum* Desf.) callus culture to osmosis- induced drought stress caused by Polyethylene glycol (PEG). Annals Biol. Res., 3(9): 4555-4563.
- EL-Meleigy, E. A.; Gabr, M. F.; Mohamed, F. H. and Ismail, M. A. (2004). Responses to NaCl salinity of tomato cultivated and breeding lines differing in salt tolerance in callus cultures. Int. J. of Agric. & Biol., 6(1): 19-26.
- Emam, A. M.; Swelam, E. S. and Megally, N. Y. (2009). Furocoumarin and quinolone alkaloids with larvicidal and antifeedant activities isolated from *Ruta chalepensis* leaves. J. Nat. Prod., 2: 10-22.
- Engelberth, J.; Schmelz, E. A.; Alborn, H. T.; Cardoza, Y.; Huang, J. and Tumlinson, J. H. (2003). Simultaneous quantification of jasmonic acid and salicylic acid in plants by vapor –phase extraction and gas chromatography – chemical ionization – mass spectrometry. Anal. Biochem., 312: 242-250.
- Errabii, T.; Gandonou, C. B.; Essalmani, H.; Abrin, J.; Idaomar, M. and Senhaji, N.S. (2007). Effects of NaCl and mannitol induced

stress on sugarcane (*Saccharum sp.)* callus cultures. Acta. Physiol. Plant, 29: 95–102.

- Faisal, M.; Ahmad, N. and Anis, M. (2005). *In vitro* regeneration and mass propagation of *Ruta graveolens* L. A-multipurpose shrub. Hortscience, 40(5): 1478-1480.
- Floridata. (2012). Http;///E:/Floridata *Ruta graveolens*.htm.
- Freire, R. B.; Borba, H. R. and Coelho, C. D. (2011). *Ruta graveolens* L. toxicity in *Vampirolepis nana* infected mice. Indian J. of Pharmacol., 42(6): 345-350.
- Fujii, H.; Verslues, P. E. and Zhu, J. K. (2007). Identification of two protein kinases required for abscisic acid regulation of seed germination, root growth, and gene expression in Arabidopsis. Plant Cell, 19: 485-494.
- Garg, G. (2010). *In vitro* screening of *Catharanthus roseus* L. cultivars for salt tolerance using physiological parameters. Int. J. Environ. Sci. Develop., 1(1): 24-30.
- Garthwaite, A.; Bothmer, R. T. and Colmer, T. D. (2005). Salt tolerance in wild *Hordium* species is associated with restricted entry of Na^+ and Cl^- into the shoots. J. Expt. Bot., 56 (419): 2365-2378.
- Gaspar, T.; Kevers, C.; Penel, C.; Greppin, H.; Reid, D. and Thorpe, T. (1996). Plant hormones and plant growth regulators in plant tissue culture. *In Vitro* Cell Dev. Biol. Plant, 32: 272-289.
- Gunaydin, A. and Savci, S. (2005). Phytochemical studies on *Ruta chalepensis* (Lam.) Lamarck. Nat. Prod. Res., 19(3): 203-210.
- Gurudeeban, S.; Satyavani, K.; Ramanathan, T. and Balasubramanian, T. (2011). Effect of antioxidant and anti-

aggregating properties of micro-propagated plantlets of *Ruta graveolens*. Afri J Biotech., 11(6):1497-1504.

- Hachicha, M. and Abd El -Gawed, G. (2003). Aspects of salt - affected soils in Arab world. In: Sustainable Strategies for Irrigation in Salt- prone Mediterranean Region: A System Approach Proc. International Workshop, Cairo, Egypt. pp: 295-310.

- Hadi, F.; Muhammad, A.; Ali, S.; Shafiq, M.; Ullah, R. and Jan, A. U. (2014). Comparative effect of polyethylene glycol and mannitol induced drought on growth (*in vitro*) of canola (*Brassica napus*), cauliflower (*Brassica oleracea*) and tomato (*Lycopersicon esculentum*) seedlings. Inter. J. Biol., 4(9): 34-41.

- Hale, A. L., Meepagala, K. M., Oliva, A., Aliotta, G. and Duke, S. O. (2004). Phytotoxins from the leaves of *Ruta graveolens*. J. Agric. Food Chem., 52 (11): 3345-3349.
- Hamad, M. N. (2012). Isolation of rutin from *Ruta graveolens* (Rutaceae) cultivated in Iraq by precipitation and fractional solubilization. Int. J. Compr. Pharm., 3(4): 1-3.
- Hani, M. and Heidari, R. (2008). Drought- induced accumulation of soluble sugars and proline in two *maize* varieties. World Appl. Sci. J., 3(3): 448-453.
- Haque, Z.; Chakraborty, A. and Prodhan, S. H. (2013). *In vitro* screening method for drought tolerance evaluation in two rice varieties (BRRI28 and BRRI29). Int. of Sci. and Eng. Res., 4(6): 339-347.
- Hashemi, K. S. M.; Sadeghpour, H. M.; Gholampour, A. I. and Mirzaei, J. H. (2011). Survey the antifungal effect of root ethanolic

extract of *Ruta graveolens* on *Saprolegnia. spp*. Int. Conf. Biotech. Environ. Manage., 18: 19-23.

- Hassanein, A. M. (2004). Effect of relatively high concentrations of mannitol and sodium chloride on regeneration and gene expression of stress tolerant (*Alhagi graecorum*) and stress sensitive (*Lycopersicon esculentum*) plant species. Bulga. J. Plant Physiol., 30(3-4): 19-36.
- Hehmann, M.; Lukavin, R.; Ekiert, H. and Matern, U. (2004). Furanocoumarins biosynthesis in *Ammi majus* L. cloning of bergaptol O-methyltransferase. Eur. J. Biochem., 271(5): 932-940.
- Hong, Z.; Lakkineni, K.; Zhang, Z. M. and Verma, D. P. (2000). Removal of feedback inhibition of delta(1)-pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. Plant Physiol., 122(4): 1129-1136.
- Htwe, N. N.; Maziah, M.; Ling, H. C.; Zaman, F. Q. and Zain, A. M. (2011). Response of selected Malaysian rice genotype to callus induction under *in vitro* salt stress. Afric. J. Biotech. 10: 350-362.
- Hussein, E. A. and Aqlan, E. M. (2011). Effect of mannitol and sodium chloride on some total secondary metabolites of fenugreek calli cultured *in vitro*. Plant Tiss. Cult. Biotech., 21 (1): 35-43.
- Jakab, G.; Ton, J.; Flors, V.; Zimmerli, L.; Metraux, J. and Mauch-Mani, B. (2005). Enhancing Arabidopsis salt and drought stress tolerance by chemical priming for its abscisic acid responses. Plant Physiol., 139(1): 267-274
- Jogaiah, S.; Ramteke, S. D.; Sharma, J. and Upadhyay, A. K. (2014). Moisture and salinity stress induced changes in biochemical constituents and water relations of different grape rootstock cultivars. Int. J. Agron. United Kingdom. Volume 2014, Article ID 789087, 8 pages

- Kalyani, B. G. and Rao, S. (2014). Zeatin induced direct plant regeneration from cotyledon explants of cultivated tomato. World J. of Pharm and pharmac. Sci., 3(7): 1034-1040.
- Kamal, A. H. M.; Kim, K.; Shin, K. H.; Choi, J. S.; Baik, B. K.; Tsujimoto, H. Heo, H.; Park, C. and Woo, S. H. (2010). Abiotic stress responsive proteins of wheat grain determined using proteomics technique. Aust. J. of Crop Sci., 4(3): 196-208.
- Karamat, F.; Olry, A.; Doerper, S.; Vialart, G.; Ullmann, P.; Werck-Reichhart, D.; Bourgaud**,** F. and Hehn**,** A. (2012). CYP98A22, a phenolic ester 3`-hydroxylase specialized in the synthesis of chlorogenic acid as a new tool for enhancing the furanocoumarin concentration in *Ruta graveolens*. PMC Plant Biol., 12: 152.
- Karimi, N.; Ghasmpour, H. and Yari, M. (2014). Effect of Different growth regulators on callus induction and plant regeneration of *Satureja* species. Ann. Res. Rev. Biol., 4(16): 2646-2654.
- Karuppusamy, S. (2009). A review on trends in production of secondary metabolites from higher plants by *in vitro* tissue, organ and cell culture. J. Med. Plants Res., 3(13): 1222-1239.
- Khan, N. A.; Khan, M.; Asgher, M.; Fatma, M.; Masood, A. and Syeed, S. (2014). Salinity tolerance in plants: Revising the role of sulfure metabolites. J. Plant Biochem. Physiol., 2(1): 2-8.
- Kim, S.; Choi, H. K.; Kim, J. H.; Lee, H. S. and Hong, S. (2001). Effect of osmotic pressure on paclitaxel production in suspension cell culture of *Taxus chinensis*. Enzyme Microb. Tech., 28(2-3): 202-209.

- Koc, N. K.; Bas, B.; Koc, M. and Kusek, M. (2009). Investigation of *in vitro* selection for salt tolerant lines in sour orange (*Citrus aurantium* L.). Biotech., 8(1): 155-159.
- Kulkarni, M. and Phalke, S. (2009). Evaluating variability of root size system and its constitutive traits in hot pepper (*Capsicum annum* L.) under water stress. Sci. Hortic., 120: 159-166.
- Larkin, P.J. and Scowcroft, W.R. (1981) Somaclonal variation – a novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet., 60 (4): 197-214.
- Lechno, S.; Zamski, E. and Tel-Or, E. (1997). Salt stress-induced responses in cucumber plants. J. Plant Physiol., 150: 206–211.
- Leksungnoen, N. (2012). The relationship between salinity and drought tolerance in Turfgrasses and woody species. PhD Thesis. Utah State University, Logan, Utah. USA.
- Leone, A.; Costa, A.; Tucci, M. and Grillo, S. (1994). Adaptation versus shock response to polyethylene glycol- induced low potential in cultured potato cells. Physiol. Plant, 92(1): 21-30.
- Liang, P. (2002). A decade of differential display. Biotechniques., 33: 338-344.
- Liang, P. and Pardee, A.B. (1992). Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Sci., 257: 967–971.
- Lievre, K.; Hehn, A.; Tran, T. L. M.; Gravot, A. Thomasset, B.; Bourgaud, F.; and Gontier, E. (2005). Genetic transformation of the medicinal plant *Ruta graveolens* L. by an *Agrobacterium tumefaciens* – mediated method. Plant Sci., 168: 883-888.
- Lokhande, V. H. and Suprasanna, P. (2012). Prospects of halophytes in understanding and managing abiotic atress tolerance.

In: Environmental Adaption and Stress Tolerance of Plants in the Era of Climate Changes. Ahmed, P. and Prasad, M. N. V. (eds.). Springer Science & Business Media, LLC. pp: 29-56.

- Lokhande, V. H.; Nikam, T. D. and Penna, S. (2010). Biochemical, physiological and growth changes in response to salinity in callus cultures of *Sesuvium portulacastrum* L. Plant Cell. Tiss. Org. Cult., 102: 17-25.
- Lv, W. T.; Lin, B.; Zhang, M. and Hua, X. J. (2011). Proline accumulation is inhibitory to Arabidopsis seedlings during heat stress. Plant Physiol., 156(4): 1921-1933.
- Machakova, I.; Zazimalova, E. and George, E.F. (2008). Plant growth regulators I: Introduction; auxins, their analogues and inhibitors. In: Plant Propagation by Tissue Culture. George, E. F.; Hall, M. A. and Klerk, G. (eds). Springer, Dordrech, Netherlands. pp: 175-227.
- Mademba, F.; Bouchereau, A. and Larher, F. R. (2003). Proline accumulation in cultivated citrus and its relationship with salt tolerance. The J. Hort. Sci. Biotechnol., 78(5): 617–623.
- Madhava Rao, K. V. (2006). Introduction, chapter one. In: Physiology and Molecular Biology of Stress Tolerance in Plants. Madhava Rao, K. V.; Raghavendra, A. S. and Janardhan Reddy, K. J. (eds.). Springer, Netherlands. pp: 1-14.
- Mahajan, S. and Tuteja, N. (2005). Cold, salinity and drought stresses: An overview. Arch. Biochem. Biophy., 444(2): 139-158.
- Mahdi, E. M. and Idris, T. I. M. (2013). The effects of NaCl pre-treatment on salt tolerance of tomato (*Lycopersicon esculentum* Mill.) callus grown under elevated saline conditions. Int. Res. J. Biotech., 4(3): 61-67.

- Mahmood, I., Razzaq, A., Hafiz, I. A., Kaleem, S., Khan, A. A. and Ahmad, M. (2012). Interaction of callus selection media and stress duration for *in vitro* selection of drought tolerant callus of wheat. Afric. J. Biol., 11(17): 4000-4006.
- Maria, P. N. (2012). Genetic transformation and elicitation to obtain medicinal compounds in grapevine (*Vitis vinifera* L.) and in *Bituminaria bituminosa* (L.) stirt. Thesis. Department Ciencia y Technologia Agraria. Universided Politecnica de Cartagna,
- Marroquin, D. S.; Guzman, C. A. L.; Flick, A. C.; Bello, J. J. B. and Buzzy, N. S. (2011). *In vitro* selection of salt- tolerant embryogenic line in habanero pepper (*Capsicum chinense* Jacq). Hortscience, 46(12): 1666-1671.
- Massot, B., Milesi, S., Gontier, E., Bourgarud, F. and Guckert, A. (2000). Optimized culture conditions for the production of furanocoumarins by microprpagated shoots of *Ruta graveolens.* Plant Cell Tiss. Org. Cult., 62: 11-19.
- Matheka, J. M.; Magiri, E.; Rasha, A. O. and Machuka, J. (2008). *In vitro* selection and characterization of drought tolerant somaclones of tropical maize (*Zea mays* L.). Biotechnology, 7(4): 641-650.
- Mazid, M.; Khan, T. A. and Mohammad, F. (2011). Role of secondary metabolites in defense mechanism of plants. Biol. Medic., 3(2): 232-249.
- Miki, Y.; Hashiba, M. and Hisajima, S. (2001). Establishment of salt stress tolerant rice plants through step up NaCl treatment *in vitro*. Biol. Plant, 44(1): 391-395.

- Milesi, S.; Massot, B.; Gontier, F.; Bourgaud, F. and Guckert, A. (2001). *Ruta geaveolens* L.: a promising species of the production of furanocoumarins. Plant Sci., 161: 189-199.
- Missouri Botanical Garden. (2012). http:///missouribotanicalgarden.org/garden-gardens/your-garden/plant-finder/.../*Ruta-graveolens*.aspex.
- Mitoi, E. M.; Holobiuc, I. and Blindu, R. (2009). The effect of mannitol on antioxidantive enzymes *in vitro* long term cultures of *Dianthus tenuifolius* and *Dianthus spiculifolius*. Rom. J. Biol. Plant Biol., 54(1): 25-33.
- Mohamed, M. A. and Ibrahim, T. A. (2012). Enhanced *in vitro* production of *Ruta graveolens* L. coumarins and rutin by mannitol and ventilation . Plant Cell Tiss. Org. Cult., 111: 335-343.
- Mok, D. W. and Mok, M. C. (2001). Cytokinin metabolism and action. Ann. Rev. of Plant Physoil. Plant Mol. Biol., 52: 89-118.
- Moshkov, I. E.; Novikova, G.V.; Hall, M.A. and George, E.F. (2008). Plant growth regulators III: Gibberellins, ethylene, abscisic acid, their analogues and inhibitors; Miscellaneous compounds. In: Plant Propagation by Tissue Culture. George, E. F.; Hall, M. A. and Klerk, G. (eds). Springer, Dordrech, Netherlands. pp: 227-283.
- Muller, E., Brown, P.T.H., Hartke, S. and Lorz, H. (1990). DNA variation in tissue-culture-derived rice plants. Theor. Appl. Genet., 80: 673-679.
- Munns, R. and Tester, M. (2008). Mechanisms of salinity tolerance. Annu. Rev. Plant Biol., 59: 651–681.

- Murashige, T. and Skoog. (1962). A revised medium for rapid growth and bioassays with Tobacco tissue culture. Physiol. Plant, 15: 473-497.
- Muszynska, A.; Jarocka, K. and Kurczynska, E. U. (2014). Plasma membrane and cell wall properties of an aspen hybrid (*Populus tremula x tremuloides)* parenchyma cells under the influence of salt stress. Acta Physiol. Plant, 36: 1155-1165.
- Nawaz, S.; Ahmed, N.; Iqbal, A. and Khaliq, I. (2013). Optimization of regeneration protocols for wheat under drought and salt stress. Pak. J. Agric. Sci., 50(4): 663-670.
- Ndagijimana, V.; Kahia, J.; Asiimwe, T.; Sallah, P. Y.; Waweru, B.; Mushimiyimana, I.; Ndirigwe, J.; Kirimi, S.; Shumnusha, D.; Njenga, P.; Kouassi, M. and Koffi, E. (2014). *In vitro* effects of gibberellic acid and sucrose concentration on micropropagation of two elite sweet potato cultivars in Rawanda. Int. J. Biotech. Mol. Biol. Res., 5(1): 1-6.
- Oliva, A.; Meepagala, K. M.; Wedge, D. E.; Harries, D.; Hale, A.; Aliotta, G.; Duke, S. and Duke, S. O. (2003). Natural fungicides from *Ruta graveolens* L. leaves, including a new quinolane alkaloids. J. Agric. Food Chem., 51(4): 890-896.
- Oraibi, A. K. (2013). *In vitro* and *in vivo* investigation of DNA marker for drought tolerance in some rice (*Oryza sativa* L.) genotypes. PhD Thesis, College of Science, University of AL-Nahrain, Baghdad, Iraq.
- Orlita, A.; Sidwa-Gorycka, M. S.; Kumirska, J.; Malinski, E. Siedlecka, E.; Gajdus, J.; Lojkowska, E. and Stepnowski, P. (2008). Identification of *Ruta graveolens* L. metabolites

accumulated in the presence of abiotic elicitors. Biotechnol. Prog., 24: 128-133.

- Pan, Y.; Wu, L. J. and Yu, Z. L. (2006). Effect of salt and drought stress on antioxidant enzymes activities and SOD isoenzymes of liquorice (*Glycyrrhiza uralensis* Fisch). Plant Growth Regul., 49: 157–165.
- Panghal, S. and Soni, S. S. (2014). *In vitro* studies on effect of different concentration of NaCl on *Jatropha curcas*. J. Environ. Biol., 35: 709-712.
- Pare, P.W. and Tumlinson, J. H. (1999). Plant volatiles as a defense against insect herbivores. Plant Physiol., 121: 325–332.
- Patade, V. Y.; Suprasanna, P.; Bapat, V. A., and Kulkarni, U. G. (2006). Selection for abiotic (salinity and drought) stress tolerance and molecular characterization of tolerant lines in sugarcane. BARC Newsletter, 273: 244-257.
- Peisheng, C.; Narendra, S.; Joe, C.; Robert, L. (2004). Differentially expressed genes during drought and heat stress in cotton (*Gossypium hirsutum*). Genomic and proteomic resources. Poster: Genomic & proteomic resources. Plant Biology 2004: Friday, July 24 - Wednesday July 28, 2004. Lake Buena Vista, FL., USA.
- Peleg, Z., Apse, M. P. and Blumwald, E. (2011). Engineering salinity and water – stress tolerance in crop plants: Getting closer to the field. Adv. Bot. Res., 57: 405-443.
- Perez- Clemente, R. M. and Gomez – Cadenas, A. (2012). *In vitro* tissue culture, a tool for the study and breeding of plants subjected to abiotic stress. In: Recent Advances in Plant *In Vitro* Culture. Leva, A. and Rinaldi, M. (eds.). InTech Publisher. pp: 91-108.

- Placide, R.; Christian, C. S. and Rony, S. (2012). Development of *in vitro* technique to screen for drought tolerant banana varieties by sorbitol induced osmotic stress. Afric. J. Plant Sci., 6(15): 416-425.
- Preethi, K. C.; Kuttan, G. and Kuttan, R. (2006). Anti – tumour activity of *Ruta graveolens* extract. Asia. Pac. J. Cancer Prev., 7: 439-443.
- Preethi, K. C.; Nair, C. K. K.; Kuttan, R. (2008). Clastogenic potential of *Ruta graveolens* extract and a homeopathic preparation in mouse bone marrow cells. Asia. Pac. J. Cancer Prev., 9: 763-769.
- Purohit, M.; Srivastava, S. and Srivastava, P. S. (1998). Stress tolerant plants through tissue culture. In: Plant Tissue Culture and Molecular Biology: Application and Prospects. Srivastava, P. S. (ed.). Narosa Publishing House, New Delhi, pp: 554–578.
- Qin, F.; Shinozaki, K. and Yamaguchi-Shinozaki, K. (2011). Achievements and challenges in understanding plant abiotic stress responses and tolerance. Plant Cell Physiol., 52(9): 1569–1582.
- Queiros, F.; Fidalgo, F.; Santos, I. and Salema, R. (2007). *In vitro* selection of salt tolerant cell lines in *Solanum tuberosum* L. Biol. Plantarum., 51(4); 728-734.
- Rai, M. K.; Kalia, R. K.; Singh, R.; Gangola, M. and Dhawan, A. K. (2011). Developing stress tolerant plants through *in vitro* selection – An overview of the recent progress. Environ. Exp. Botany., 71: 89-98.
- Rajeswari, S.; Krishnamurthi, M.; Shinisekar, S., Prem Anand, S. and Thirugnana Kumar, S. (2009). Performance of somaclones developed from intergeneric hybrids of sugarcane. Sugar Tech., 11(3): 258- 261.

- Ramakrishna, A. and Ravishankar, A. (2011). Influence of abiotic stress signals on secondary metabolites in plants. Plant Signal. Behav., 6(11): 1720-1731.
- Ramawat, K. G. (2008). Plant Biotechnology. S. Chand & Company LTD. Ram Nagar, New Delhi. pp: 32-36.
- Rao, S. and Patil, P. (2012). In vitro selection of salt tolerant calli lines and regeneration of salt tolerant plantlets in mung bean (*Ving radiate*). Molecular Studies and Novel Applications for Improved Quality of Human Life, 1: 197-212.
- Ratheesh, M. and Helen, A. (2007). Anti-inflammatory activity of *Ruta graveolens* Linn on carrageenan induced paw edema in wistar male rats. Afric. J. Biotechnol., 6(10): 1209-1211.
- Raveendar, S.; Premkumar, A.; Ignacimuthu, S. and Agastian, P. (2008). Effect of sea water on callus induction and regeneration of rice genotype. Int. J. of Integr. Biol., 3(2): 92-95.
- Razmjoo, K.; Heydarizadeh, P. and Sabzalian, M. R. (2008). Effect of salinity and drought stress on growth parameters and essential oil content of *Matricaria chamomila*. Int. J. Agric. Biol., 10: 451-454.
- Rehem, B. C., Bertolde, F. Z. and de Almeida, A. (2012). Regulation of gene expression in response to abiotic stress in plants. In: Cell Metabolism - Cell Homeostasis and Stress Response. Bubulya, B. (ed.). InTech Publisher. pp: 13-38.
- Rodriguez, M.; Canales, E. and Borrás-Hidalgo, O. (2005). Molecular aspects of abiotic stress in plants. Biotecnología Aplicada., 22: 1-10.
- Sakpere, A. M. A.; Ajayi, S. A. and Adelusi, A. A. (2014). Effect of growth regulators and explants types on callus induction in

Telfairia occidentalis Hook F.. Afric. J. Biotech., 13(20): 2015-2021.

- Sakthivelu, G.; Akitha Devi, M. K.; Giridhar, P.; Rajasekaran, T.; Ravishankar, G. A.; Nedev, T. and Kosturkova, G. (2008). Drought induced alteration in growth, osmotic potential and *in vitro* regeneration of soybean cultivars. Gen. Appl. Plant Physiol., 34(1-2): 103-112.
- Sen, A. (2012). Oxidative stress studies in plant tissue culture. In: Antioxidant Enzyme. El-Missiry, M. (ed.). InTech publisher. ISBN: 978-953-51-0789-7, InTech, DOI: 10.5772/48292. Available from: http://www.intechopen.com/books/antioxidant-enzyme/oxidative-stress-studies-in-plant-tissue-culture.
- Shabana, M.M. ; El-Alfy, T.S. ; El-Tantawy, M.E. ; Ibrahim, A.I. and Ibrahim, G.F. (2001). Tissue culture and evaluation of some active constituents of Ruta graveolens L. I: Effect of plant growth regulators and explant type on growth of Ruta graveolens L. callus cultures. Arab J. Biotech., 4(2):
- Shankhdhar, D.; Shankhdhar, S. C., Mani, R. and Pant, R. C. (2000). In vitro selection of salt tolerance in rice. Biol. Plant, 43(1): 477-480.
- Sharma, V. and Ramawat, K. G. (2013). Salinity-induced modulation of growth and antioxidant activity in the callus cultures of miswak (*Salvadora persica*). 3Biotech., 3 (1): 11-17.
- Shehab, G. G.; Ahmed, O. K. and EL-Beltagi, H. S. (2010). Effects of various chemical agents for alleviation of drought stress in rice plants (*Oryza sativa* L.). Not. Bot. Hort. Agrobot. Cluj., 38(1): 139-148.

- Shehadeh, M. B.; Afifi, F. U. and Abu-Hamdah, S. M. (2007). Platelet aggregation inhabitions from aerial parts of *Ruta chalepensis* grown in Jordan. Integrative Med. Insights, 2: 35-39.
- Shomeli, M.; Nabipour, M.; Meskarbashee, M. and Memari, H. (2011). Evaluation of sugarcane (*Saccharum officinarum* L.) somaclonals tolerance to salinity via *in vitro* and *In vivo*. Hayati J. Biosci., 18 (2): 91-96.
- Sidwa-Gorycka, M. S.; Krolicka, A.; Kozyra, M.; Glowniak, K.; Bourgaud, F. and Lojkwska, E. (2003). Establishment of a co – culture of *Ammi majus* L. and *Ruta graveolens* L. for the synthesis of furanocoumarin. Plant Sci., 165 (6): 1315-1319.
- Sidwa-Gorycka, M. S.; Krolicka, A.; Orlita, A.; Malinski, E.; Golebiowski, M. and Lojkowska, E. (2009). Genetic transformation of *Ruta graveolens* L. by *Agrobacterium rhizogenes*: hairy root cultures a promising approach for production of coumarins and furanocoumrins. Plant Cell Tiss. Org. Cult., 97: 59-69.
- Sofic, E.; Copra-Janicijevic, A.; Salihovic, M.; Tahirovic, M and Kroyer, G. (2010). Screening of medicinal plant extract for quercetin-3-rutinoside (rutin) in Bosnia and Herzegovina. Medicinal Plants-International Journal of Phytomedicines and Related Industries, 2(2): 97-102.
- Srivastarva, J. and Raghav, P. K. (2014). Effect of different gibberellic acid concentration on pigeonpea (Cajanus cajan [L.] Millsp.) cv. Manak (H77216) via cotyledonary node explants. Int. J. Adv. Biotech. Res., 5(2): 151-156.

- Steel, R. and Torrie, J. (1982). Principles and Procedures of Statistics, 2nd. McGraw-Hill International Book Company. Auckland, London.
- Stoeva, N. and Kaymakanova, M. (2008). Effect of salt stress on the growth and photosynthesis rate of bean plants (*Phaseolus vulgaris* L.). J. Cent. Eur. Agr., 9 (3): 385–392.
- Summart, J.; Thanonkeo, P.; Panichajakul, S.; Prathepha, P. and McManus, M. T. (2010). Effect of salt stress on growth, inorganic ion and proline accumulation in Thai aromatic rice, Khao Dawk Mali 105, callus culture. Afric. J. Biotech., 9(2): 145-152.
- Szewczyk, K. and Bogucka-Kocka, A. (2012). Analytical methods of isolation, separation and identification of selected furanocoumarins in plant material. In: Agricultural and Biological Sciences "Phytochemicals –A Global Perspective of Their Role in Nutrition and Health". Venketeshuer, R. (ed.). InTech Publisher. PP: 57-92.
- Taha, R. A. (2014). Effect of growth regulators and salinity levels on *in vitro* cultures of Jojoba plants. World App. Sci. J., 31(5): 751-758.
- Taha, R. A. and Hassan, M. M. (2014). Using low levels of sea water to enhance growth and development of date palm embryogenic cultures. Asian. J. Agric. Sci., 6(2): 69-74.
- Tamirisa, S.; Vudem, D. R. and Khareedu, V. R. (2014). Overexpression of pigeonpea stress-induced cold and drought regulatory gene (*CcCDR*) confers drought, salt, and cold tolerance in *Arabidopsis.* J. Exp. Botany, 65 (17): 4769-4781.

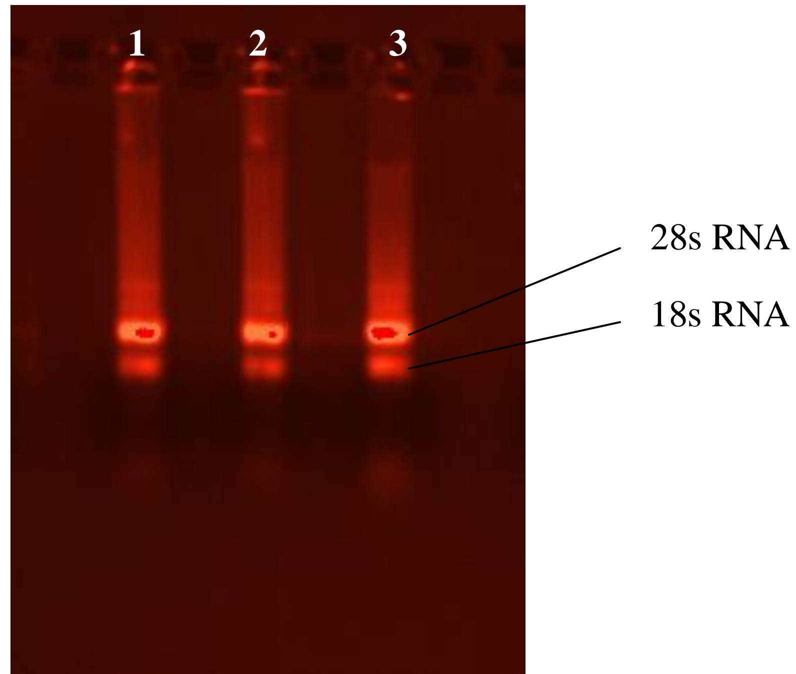
- Tang, W. and Newton, R. J. (2004). Increase of polyphenol oxidase and decrease of polyamines correlate with tissue browning in Virginia pine (*Pinus virginiana,* Mill.). Plant Sci., 167: 621-628.
- Tariq, M.; Ali, G.; Hadi, F.; Ahmed, S.; Ali, N. and Shah, A. (2008). Callus induction and *in vitro* plant regeneration of rice (*Oryza sativa* L.) under various conditions. Pak. J. Biol. Sci., 11(2): 255-259.
- Terryn, N.; Van Montagu, M.; Inzé, D. and Goossens, A. (2006). Functional genomic approaches to study and engineer secondary metabolism in plant cell cultures. Frontis, 17:291-300.
- Tong, S.; Ni, Z.; Peng, H.; Dong, G. and Sun, Q. (2007). Ectopic overexpression of wheat *TaSrg6* gene confers water stress tolerance in Arabidopsis. Plant Sci., 172 (6): 1079–1086.
- Torres, G. A. M.; Gimenes, M. A.; de Rosa, J. and Quecini, V. (2007). Identifying water stress-response mechanisms in citrus by in silico transcriptome analysis. Genetic Molecul. Biol., 30(3): 888-905.
- Tripathi, A.; Aggarwal, R. and Yadav, A. (2013). Differential expression analysis of defense -related genes responsive to *Tilletia indica* infection in wheat. Turk. J. Biol., 37: 606-613.
- Tumbas, V.; Mandić, A.; Ćetković, G.; Đilas, S. and Čanadanović-Brunet, J. (2004). HPLC analysis of phenolic acids in mountain germander (*Teucrium montanum* L.) extracts. Acta Periodica Technologica, 35(1): 265-273.
- USDA, GRIN Taxonomy for Plants. http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?32578#common.
- Vanisree, M.; Lee, C.; Lo, S.; Nalawade, S.; Lin, C. and Tsay, H. S. (2004). Studies on the production of some important secondary

metabolites from medicinal plants by plants tissue cultures. Bot. Bull. Acad. Sci., 45: 1-22.

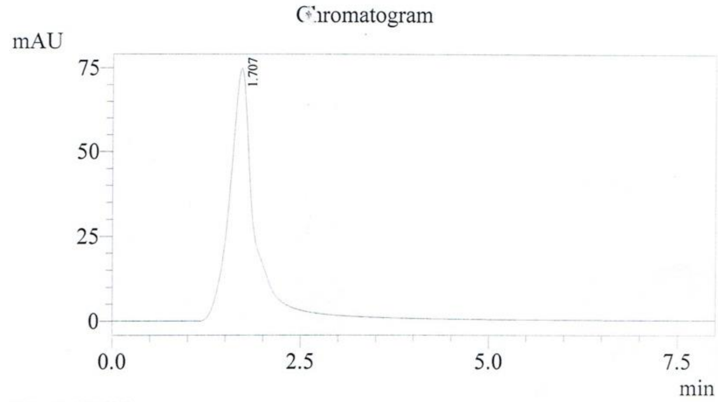
- Varbanova, K.; Dimitrova, D. and Kornova, K. (2005). Growth and development of *Ruta graveolens* L. depending on the method of multiplication. In: Proceeding of the Balkan Scientific Conference of Biology in Plovdiv (Bulgaria) from 19th Till 21th of May 2005. pp: 289-293.
- Vazquez – Flota, F. A. and Loyola – Vargas, V. M. (1994). A *Catharanthus roseus* salt tolerant line selection and characterization. J. Plant Physiol., 144: 116-120.
- Vázquez, A. M. and Linacero, R. (2010). Stress and Somaclonal Variation. In: Plant Developmental Biology – Biotechnological Perspectives. Pua, E. C. and Davey, M. R. (eds.). Springer – Verlag Berlin Heidelberg. pp: 5-64.
- Vogelstein, B. and Gillespie, D. (1979). Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA, 76(2): 615-619.
- Waditee, R.; Tanaka, Y. and Takabe, T. (2006). Na^+/H^+ antiporter in plants and *cyanobacteria*. In: Abiotic Stress Tolerance in Plants, Toward the Improvement of Global Environment and Food. Rai, A. K. and Takabe, T. (eds.). Springer, Dordrecht, The Netherlands. pp: 161-177.
- Wani, S. H.; Singh, N. B.; Haribhushan, A. and Mir, J. I. (2013). Compatible solute engineering in plants for abiotic stress tolerance - role of glycine betaine. Curr. Genomics, 14(3): 157-165
- Wani, S. H.; Sofi, P.; Gosal, S. and Singh, N. (2010). *ln vitro* screening of rice (*Oryza sativa* L.) callus for drought tolerance. Comm. in Bio. Crop Sci., 5(2): 108-115.

- Wink, M. (2010). Occurrence and function of natural production in plants. Phytochemistry and Phrmacognosy. Encyclpedia of life Support Systems (EOLSS).
- Xiong, L. and Ishitani, M. 2006. Stress signal transduction: components, pathways and network integration In: Abiotic Stress Tolerance in Plants: Toward the Improvement of Global Environment and Food. Rai, A.K. and Takabe, T. (ed). Springer Publishers, Dordrecht, The Netherlands. pp. 3-29.
- Yousif, SH. A. 2002. Evaluation and regeneration salt tolerant rice plant using different techniques. PhD thesis, Crop Sci., Dept., Agricultural Collage. Baghdad University.
- Zhao, J.; Hu, Q.; Guo. Y. Q. and Zhu, W. H. (2001). Effects of stress factors, bioregulators, and synthetic precursors on indole alkaloid production in compact callus clusters cultures of *Catharanthus roseus*. Appl. Microb. Biotech., 55: 693–698.
- Zhu, J. K. (2001). Plant salt tolerance. Trends Plant Sci., 6 (2): 66-71.
- Ziegler, H. (1990). Role of plant physiology in assessing potential under stress environment. Proceedings of the lnternational Congress of Plant Physiology, New Delhi, lndia, 88: 10-17.
- Ziv, M. and Chen, J. (2008). The Anatomy and Morphology of Tissue Cultured Plants. In: Plant Propagation by Tissue Culture. George, E. F.; Hall, M. A. and Klerk, G. (eds). Springer, Dordrech, Netherlands. . pp: 465-479.
- Zuraida, A. R.; Shukri, M.; Sabrina, E.; Izzati, F.; Nazreena, A., Juhazliana, J. and Zaliha,W. (2014). Improved plant regeneration and *in vitro* somatic embryogenesis in *Ruta graveolens*. J. Exp. Biol. Agric. Sci., 2(3): 328-336.

Appendices



Appendix (1): Analysis of RNA, (1): RNA isolated from regenerated non stressed plantlet, (2 and 3): RNA isolated from regenerated stressed plantlets, RNA sample was loaded on 1% agarose gel stained with ethidium bromide.



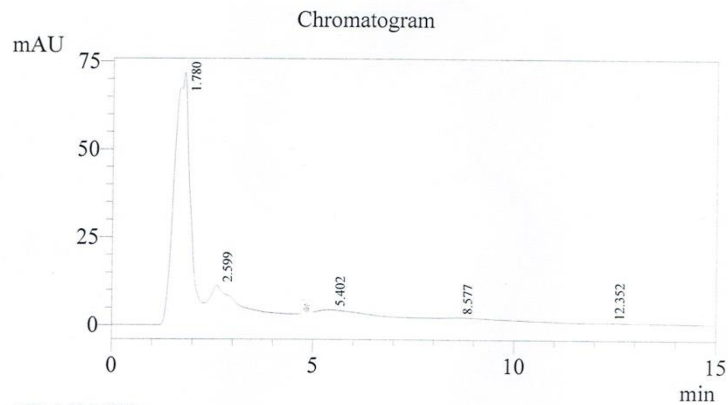
1 Det.A Ch1 / 300nm

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %
1	1.707	1854546	74861	100.000
Total		1854546	74861	100.000

(A)



1 Det.A Ch1 / 300nm

PeakTable

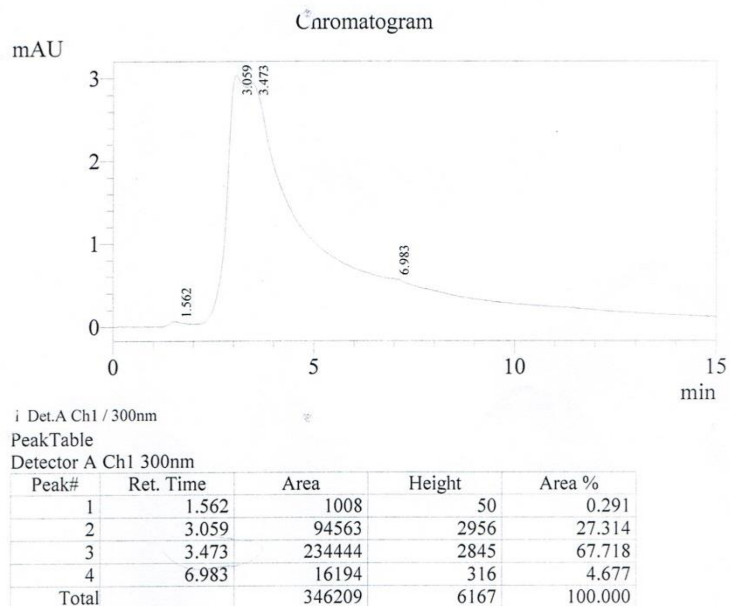
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %
1	1.780	1986134	71674	66.759
2	2.599	658233	10745	22.125
3	5.402	308143	3000	10.358
4	8.577	19327	240	0.650
5	12.352	3225	94	0.108
Total		2975061	85753	100.000

(B)

Appendix (2): (A) HPLC chromatogram of the standard gallic acid.

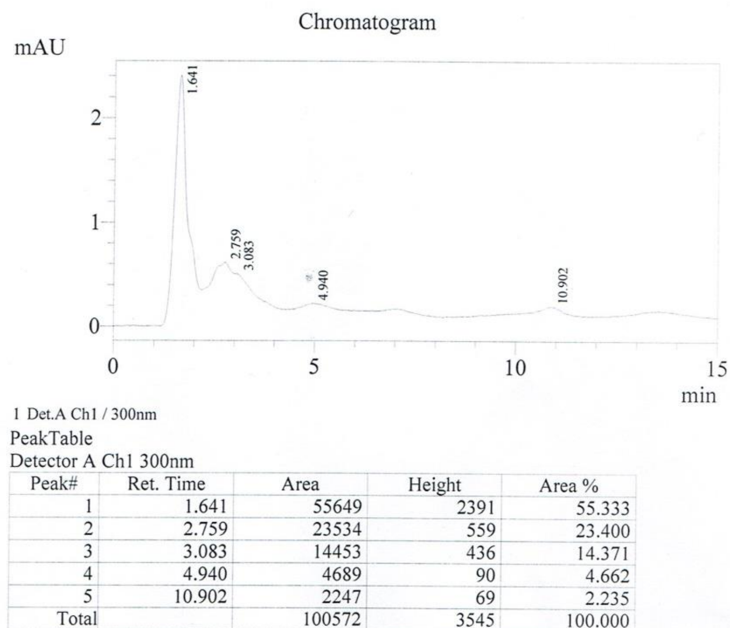
(B) HPLC chromatogram of the extracted gallic acid.



PeakTable
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %
1	1.562	1008	50	0.291
2	3.059	94563	2956	27.314
3	3.473	234444	2845	67.718
4	6.983	16194	316	4.677
Total		346209	6167	100.000

(A)



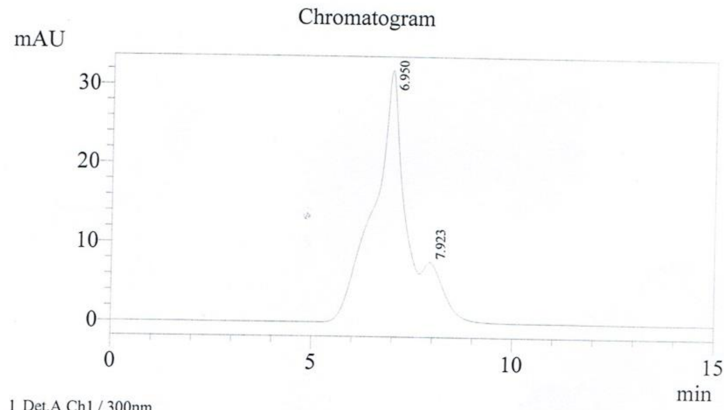
PeakTable
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %
1	1.641	55649	2391	55.333
2	2.759	23534	559	23.400
3	3.083	14453	436	14.371
4	4.940	4689	90	4.662
5	10.902	2247	69	2.235
Total		100572	3545	100.000

(B)

Appendix (3): (A) HPLC chromatogram of the standard rutin.

(B) HPLC chromatogram of the extracted rutin.



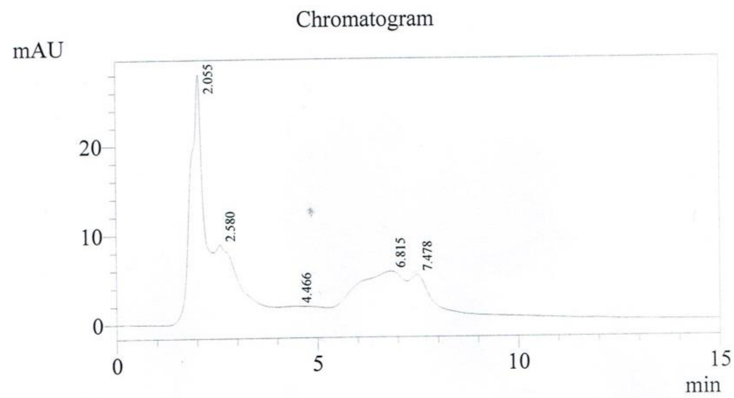
1 Det.A Ch1 / 300nm

PeakTable

Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %
1	6.950	1648538	31967	84.350
2	7.923	305871	7687	15.650
Total		1954408	39653	100.000

(A)



1 Det.A Ch1 / 300nm

PeakTable

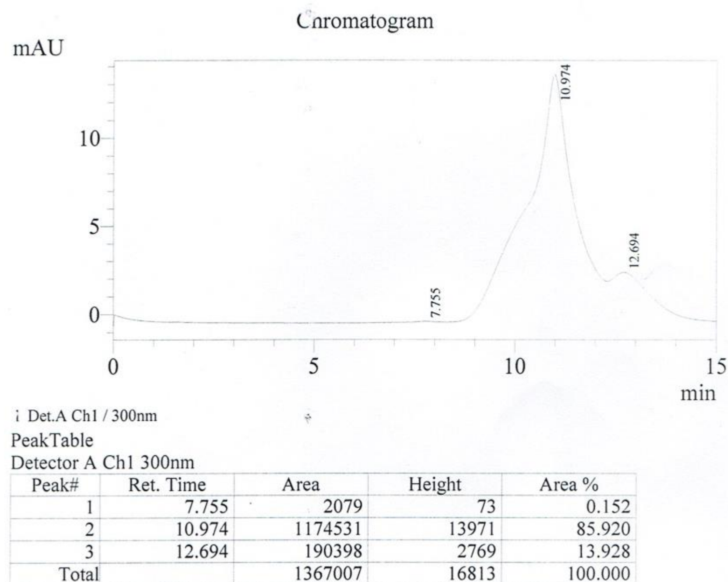
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %
1	2.055	648573	28112	33.056
2	2.580	434364	8877	22.138
3	4.466	129211	1843	6.585
4	6.815	483305	5550	24.633
5	7.478	266603	5108	13.588
Total		1[illegible]52056	49490	100.000

(B)

Appendix (4): (A) HPLC chromatogram of the standard xanthotoxin.

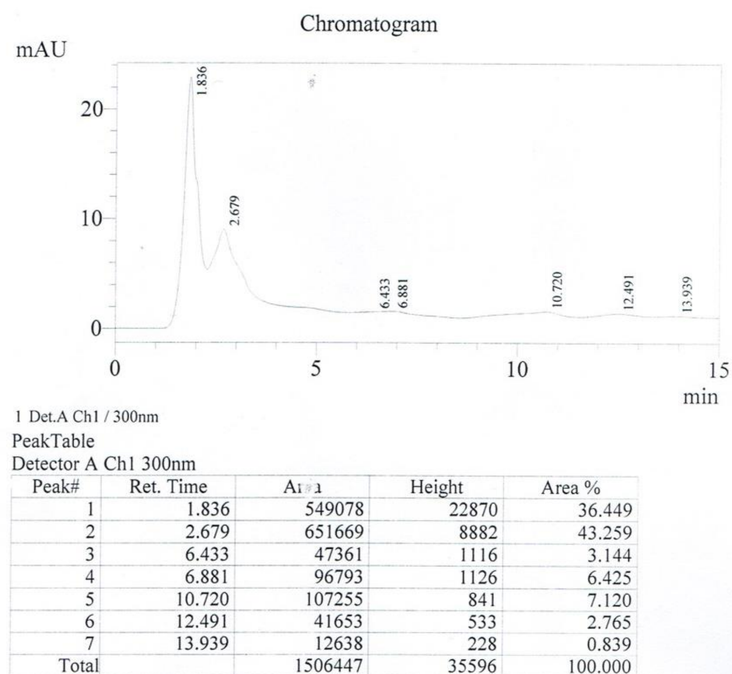
(B) HPLC chromatogram of the extracted xanthotoxin.



PeakTable
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %
1	7.755	2079	73	0.152
2	10.974	1174531	13971	85.920
3	12.694	190398	2769	13.928
Total		1367007	16813	100.000

(A)



PeakTable
Detector A Ch1 300nm

Peak#	Ret. Time	Area	Height	Area %
1	1.836	549078	22870	36.449
2	2.679	651669	8882	43.259
3	6.433	47361	1116	3.144
4	6.881	96793	1126	6.425
5	10.720	107255	841	7.120
6	12.491	41653	533	2.765
7	13.939	12638	228	0.839
Total		1506447	35596	100.000

(B)

Appendix (5): (A) HPLC chromatogram of the standard bergapten.

(B) HPLC chromatogram of the extracted bergapten.

الملخص

ُيفيد تشخيص الصفات الوظيفية والتركيبية للجينات المسؤولة عن تحمل الاجهاد البيئي الى فهم أفضل لآلية عمل النباتات في تحملها لمختلف الأجهادات اللاحيوية. ُوظفت تقانة زراعة الآنسجة النباتية للحصول على نباتات متحملة للملوحة والجفاف كما وظفت تقانة العرض التفريقي DDRT-PCR للكشف عن الفروقات أو الأختلافات بين الجينات لنبيتات السذاب *Ruta graveolens* المعرضة وغير المعرضة لأجهادي الملوحة والجفاف. بلغت أعلى نسبة لإستحثاث الكالس من أوراق نبات السذاب 96.3% بأضافة 1.5 ملغم/لترمن 2,4-D و0.5 ملغم/لترKin الى الوسط الزرعي MS، بينما أستحث الكالس من سيقان النبات بأعلى نسبة بلغت 100% بأضافة 1 ملغم /لتر 2,4-D و Kin.ُسجلت أعلى نسبة مئوية لإكثار الكالس بوجود 1 ملغم/لتر 2,4-D و 0.5 ملغم/لتر و Kin. سجل أفضل معدل للأستحثاث بعد تجهيز الوسط بالتراكيز 1.5 ملغم/لترBA و0.5 ملغم/لتر NAA.ُحفزت النموات الخضرية على الأستطالة بإضافة تراكيز مختلفة من حامض الجبرليك GA_3 إلى الوسط الزرعي MS. أظهرت النتائج أن أعلى معدل للاستطالة كان بأضافة التركيز0.5 ملغم/لتر. جذرت النموات الخضرية وتم أقلمتها بنقلها الى الوسط الزرعي MS بنصف القوة مدعم بحامض اندول البيوترك بالتركيز0.1 ملغم/لتر. أظهرت النتائج حصول نسبة تجذير 100% للنموات الخضرية. ُنفذت تجارب عدة لغربلة خلايا كالس نبات السذاب المتحملة لعاملي الإجهاد الجفافي المانتول و الملحي بإستعمال ماء البزل بالطريقتين المباشرة والتدريجية، ثم ُقيم تأثير هذين العاملين على المستوى الخلوي.ُعرضت خلايا الكالس وبشكل مباشر الى تراكيز مختلفة من المانتول (0.0-300)غم/لتر واظهرت النتائج إن التعرض المباشر للإجهاد الرطوبي أدى إلى إنخفاض ملحوظ في الوزن الطري للكالس.ُسجل أعلى وزن طري للكالس 188غم عند التعرض لتركيز 240غم/لتر مانتول ولفترة تعرض دامت 12اسبوعاً.ُغربلت خلايا الكالس بطريقة تدريجية بتعريضها الى تراكيز (0.0-300)غم/لتر من المانتول.ُسجل اعلى معدل وزن طري للكالس في التركيز 300 غم/لتر مانتول بلغ 281غم بعد النقلة الخامسة للكالس وبعمر20 اسبوع.ُعرض الكالس وبشكل مباشر الى تراكيز مختلفة من ماء البزل (5.0-30.0) ديسيمنز/م$^{-1}$،ُسجلت زيادة معنوية في معدل نمو الكالس عند تعرضه للإجهاد الملحي في التركيز 25 ديسيمنز/م$^{-1}$ من ماء البزل ولمدة 12 اسبوع بلغ 233غم مقارنة بكالس السيطرة.ُعرض الكالس الى تراكيز تدريجية من ماء البزل تراوحت من 5.0-30 ديسيمنز/م$^{-1}$ وأظهرت النتائج عدم وجود اي فروقات معنوية في معدل وزن الكالس بين التراكيز الملحية مقارنة بكالس السيطرة، اما أعلى معدل لوزن الكالس فكان 644 غم في التركيز 30 ديسيمنز/م$^{-1}$ ولمدة 20 اسبوع. أوضحت النتائج ان النسبة المئوية

للإخلاف اختلفت معنويا في كل المعاملات المتعرضة لإجهادي الملوحة والجفاف بالمقارنة مع معاملة السيطرة. حقق الكالس المعرض للمانتول 300 غم/لتر بطريقة تدريجية أعلى نسبة للإخلاف وصلت الى 85.03%، في حين وصلت النسبة المئوية الى 64.31% في خلايا الكالس المعرضة للملوحة بطريقة تدريجية. إزداد تركيز البرولين في النموات المستحثة من الكالس المعرض للمانتول و ماء البزل بالطريقتين المباشرة والتدريجية. حصلت زيادة ملحوظة في محتوى حامض الجالك في كافة النباتات المتعرضة للإجهاد بالطريقتين المباشرة والتدريجية مقارنة مع نباتات السيطرة، 22.6ملغم/غم وزن طري التركيزالأعلى للحامض وجد في النباتات المتعرضة لإجهاد الملوحة بشكل مباشر. زاد التعرض المباشر للإجهادين من محتوى مركب الروتين في النموات الخضرية مسجلا 311 ملغم/غم وزن طري وهي اعلى قيمة له في النموات المتعرضة بشكل مباشر لإجهاد الملوحة. إزداد تركيز الزانثوتوكسين الى 12.3 و11.3 ملغم/غم وزن طري في النموات المستحثة من الكالس المتعرض بشكل مباشر لإجهاد الملوحة وبشكل تدريجي لإجهاد الجفاف على التوالي، أعلى تركيز للبركابتين كان 2.29 ملغم/غم وزن طري في النموات المستحثة من الكالس المتعرض للمانتول بشكل مباشر. حققت النباتات المستحثة من الكالس المعرض سابقا للملوحة بالطريقتين المباشرة والتدريجية تحملا للملوحة بنسبة 75 و 80% على التوالي بعد التعرض المباشر لماء البزل لمدة 40 يوم. اما النباتات المستحثة من الكالس المعرض سابقا للجفاف بالطريقتين المباشرة والتدريجية فأظهرت تحملا للجفاف بنسبة 70 و 72% على التوالي بعد التعرض المباشر للمانتول لمدة 40 يوم. أظهرت تقنية العرض التفريقي إختلافات بين التعبير الجيني للنباتات المعرضة وغير المعرضة لإجهادي الملوحة والجفاف لنباتات السذاب باستخدام البرايمرات OPA-01،OPA-08, OPA-11 وOPA-17 التي اظهرت زيادة التعبير للجينات او تفعيل جينات جديدة بفعل التعرض لإجهادي الملوحة والجفاف.

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تأثير الآجهاد اللاأحيائي في تراكم بعض مركبات الآيض الثانوي في نبات *Ruta graveolens* خارج الجسم الحي

اطروحة

مقدمة الى كلية العلوم/ جامعة النهرين
وهي جزء من متطلبات نيل درجة دكتوراه فلسفه في التقانة الاحيائية

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2014