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Enhancement of Salinity Tolerance in *Dianthus caryophyllus* L. (omaggio) using *In vitro* Selection Technique and Isolation of Potential Salinity Tolerance Genes from *Mesembryanthemum crystallinum* L.

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الجامعة الإسلامية- غزة
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قسم العلوم الحياتية

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بسم الله الرحمن الرحيم

DEDICATION

To my mother and my father

To my husband

To my daughters

Yara and Yomna

Nisreen Badr

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First and foremost, All praises and thanks are to **Allah**, who had blessed me the potential to complete this research work and compile this thesis.

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Enhancement of Salinity Tolerance in *Dianthus Caryophyllus* L. (omaggio) using *In Vitro* Selection Technique and Isolation of Potential Salinity Tolerance Genes from *Mesembryanthemum crystallinum* L.

ABSTRACT

Introduction: Salinity is one of the most serious problems affecting the agricultural productivity. Carnation is a glycophyte ornamental plant of major economical value in Gaza Strip where salinity is an ever-increasing problem. Thus the development of new cultivar of carnation with more salt tolerance is considered of high interest to increase the productivity and the plant export.

Objective: The objective of this study was to produce new salt tolerance cultivar of *Dianthus caryophyllus* L. (omaggio) using *in vitro* selection technique and isolation of potential salinity tolerance genes from *Mesembryanthemum crystallinum* L.

Methodology: Total RNA was isolated from *M. crystallinum* L. leaves and amplified cloned via reverse transcriptase polymerase chain reaction (RT-PCR) with specific and non- specific primer used under conditions with variable stringency.

Nods of carnation were grown directly on media containing 0, 50, 100, 150 and 200 mM NaCl in order to select new cell lines have the ability to withstand high level of salt in *in vitro* and *ex vitro* conditions.

Results: Two genes were isolated and identified by sequence analysis as the putative thioredoxin gene, a salt tolerance gene and the serine/threonine-protein kinase EDR1 gene which is usually activated under biotic stress.

Carnation tissues were successfully selected and propagated under salinity conditions reaching 100 mM NaCl (EC 10 dS/m) in the Murashige and Skoog (MS) media. However, a final concentration of 200 mM and higher (EC 20 dS/m) was found cytotoxic for all the cells of carnation under the culture conditions.

Conclusion: The isolated salt tolerance gene (putative thioredoxin) and stress gene (serine/threonine-protein kinase EDR1) from a wild plant may be useful for later

genetic modification of carnation as well as other plants to increase their salinity tolerance.

The successful propagation of carnation nods under high concentration of salt in the culture medium represent a promising technique to create cultivars of carnation capable to grow at the salt conditions of Gaza Strip. The sustained propagation of our cultured carnation tissue under high salinity indicates that they may have undergone genetic changes enabling them to adapt to the character of salt tolerance.

Keywords: Salinity tolerance, carnation, Gaza Strip, *Mesembryanthemum crystallinum* L., NaCl *Dianthus caryophyllus* L. (omagio), putative thioredoxin, serine/threonine-protein kinase EDR1.

تحسين قدرة القرنفل على تحمل المياه المالحة باستخدام تقنية الانتخاب المخبري، وعزل الجينات المقاومة للملوحة من نبات الغاسول.

مقدمة: تعتبر الملوحة واحدة من أخطر المشاكل التي تؤثر على الإنتاج الزراعي. يعتبر نبات القرنفل من أكثر نباتات الزينة الاقتصادية في قطاع غزة، التي لا تتحمل الملوحة، حيث يعاني قطاع غزة بصورة دائمة من مشكلة تملح المياه. لذلك إنتاج صنف جديد من القرنفل بصفات جديدة (تحمل الملوحة) يعتبر من أنجع و أهم الحلول لزيادة الإنتاج و التصدير لهذا المنتج.

الهدف: هدفت الدراسة إلى إنتاج صنف جديد من نبات القرنفل قادر على تحمل المياه المالحة باستخدام تقنيات الانتخاب المخبري، كما هدفت الدراسة إلى عزل الجينات المقاومة للملوحة من نبات الغاسول.

منهجية البحث: خلال الدراسة تم عزل RNA و تحويله إلى cDNA بواسطة تقنية reverse transcriptase polymerase chain reaction (RT-PCR) باستخدام بادءات مخصصة و غير مخصصة تحت ظروف منخفضة لصالح البادءات.
كما و تم استزراع العقد النباتية من نبات القرنفل بشكل مباشر على أوساط مدعمة بتركيزات مختلفة من ملح كلوريد الصوديوم (0، 50، 100، 150، 200 ملي مولار)، لانتخاب خلايا جديدة بمواصفات جيدة تستطيع مقاومة الملوحة مخبريا و غير مخبريا.

النتائج: أظهرت النتائج نوعين من الجينات تم عزلها من نبات الغاسول ، أحدهما ينشط في الظروف البيئة الصعبة (الاجهاد الملحي) (putative thioredoxin)، و الآخر ينشط استجابة للتأثيرات الحيوية الصعبة (أمراض فيروسية، بكتيرية، إلخ) serine/threonine-protein kinase EDR1.
كما و تم خلال الدراسة الحصول على نبات قرنفل كامل بالانتخاب من الوسط المدعم بتركيز ملحي 100 ملي مولار من ملح كلوريد الصوديوم (10 dS/m) EC، بينما التركيزات العالية 200 ملي مولار من ملح كلوريد الصوديوم كانت قاتلة لجميع خلايا القرنفل.

الخلاصة: الجينان المعزولان من نبات الغاسول(putative thioredoxin) و serine/threonine-protein kinase EDR1، يعتبران ذا أهمية باستخدامها لاحقا ضمن التعديلات الجينية لنبات القرنفل أو أي نبات آخر لزيادة تحمل النبات للإجهاد الملحي.
تعتبر تقنية إنتاج نبات القرنفل عن طريق العقد مخبريا تحت ظروف إجهاد ملحي عالي، تقنية واعدة للحصول على أصناف جديدة لنبات القرنفل تتحمل الملوحة في قطاع غزة.

النتائج تشير إلى وجود تغيرات جينية للصنف الجديد من نبات القرنفل أكسبته قابلية مواجهة الإجهاد الملحي و التأقلم معه.

الكلمات المفتاحية: الإجهاد الملحي، القرنفل، قطاع غزة، الغاسول، كلوريد الصوديوم،
putative thioredoxin, serine/threonine-protein kinase EDR1.

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LIST OF ABBREVIATIONS

%	Percent
$	Dollar
?	Question mark
<	Above
>	Less
° ”	Degree and Second
°C	Celsius degree
2,4-D	2,4-dichlorophenoxy-acetic acid
2iP	6- dimethylaminopurine
ABA	Abscisic acid
ABI2	ABA insensitive 2
APX	Ascorbate peroxidase
BA	6- Benzyladenine
BAP	6-benzyloaminopurine
C3	Carbon three plants
Ca^{2+}	Calcium ion
CAM	Crassulacean acid metabolism
CASPP	Committee of the American Society of Plant Physiologists
Cat. No.	Catalog number
cDNA	Complementary Deoxyribonucleic acid
CDPKs	Calcium-dependent protein kinases
Chl	Chlorophyll
Cl^-	Chloride ion
CMWU	Coastal Municipalities Water Utility
CO_2	Carbon Dioxide
CRK36	Cysteine- Rich RLK
DNA	Deoxyribonucleic acid
$dS.m^{-1}$	deciSiemens per metre
e.g.	Example
EC	Electrical Conductivity
EDR 1	Enhanced Disease Resistance
FAO	Food and Agriculture Organization
Fd	Ferredoxin
g	Gram
GHR1	Guard Cell Hydrogen Peroxide- Resistant1
GMO	Genetically modified organism
H^+	Hydrogen ion
H^+-ATPase (V- ATPase)	Vacuolar-type H^+- ATPase (vacular - Adenosine triphosphatase
H_2O	Water (2 hydrogens and 1 oxygen)
H_2O_2	Hydrogen peroxide
HCO_3^-	bicarbonate
IAA	Indole Acetic Acid
IBA	indole-3- butric acide
K^+	Potassium
Kg	Kilogram

km^2	Square kilometer
L.	Linus
LRR-RLKs	Leucine-Rich Repeat Receptor-Like Kinase1
m^3	Cubic meter
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
mg/L	Milli gram per Liter
Mg^{2+}	Magnesium ion
mm	Millimetres
Mm	Micro molar
mM	Millimolar
mmol/L	Millimoles per Liter
mRNA	Messenger Ribonucleic Acid
MS	Murashige and Skoog media
MSR	methionine sulfoxide reductase
Na^+	Sodium ion
NAA	Naphthalene- acetic acid
NaCl	Sodium Chloride
NADPH	Nicotinamide Adenine Dinucleotide Phosphate Hydrogen
NCBI	National Center for Biotechnology Information
NTR	NADPH-dependent thioredoxin reductase
$O_2^{\bullet}$	Superoxide
PAR	Palestinian Agricultural Relief
PEP	Phosphoenolpyruvate
PERK4	Proline- Rich- Extensin- Like RLK4
PGR	plant growth regulator
pH	Power of hydrogen
Ph.D	Doctor of Philosophy
PPI	protein phosphatase interaction
PTC	Palestine Trade Center
PWA	Palestine Water Authority
QTL	Quantitative Trait Loci
RLKs	Rceptor-like kinases
ROS	Reactive oxygen species
RT- PCR	Reverse transcriptase- Polymerase Chain Reaction
SO_4^{-2}	Sulfate Ion
SOD	Superoxide dismutase
SOS	Salt Overly Sensitive
TAE	Tris acetate ethyldimethyl tetra acetic acid
TDZ	Thiazuron
Trxs	Thioredoxins
UV	Ultraviolet light
viz.	Videlicet which means namely
VPPase	Vacuolar pyrophosphatase
Yr	Year
β	Beta

CHAPTER 1

INTRODUCTION

INTRODUCTION

Gaza Strip is located in the semi-arid area where rainfall is falling in the winter season from mid-November to the end of April, with an average annual rainfall 335mm/year in the north, (Ubeid, 2011; CMWU, 2011). "Groundwater aquifer is considered the main and only water supply source for all kind of human usage in the Gaza Strip (domestic, agricultural and industrial)", (CMWU, 2011).This source has been affected by seawater intrusions, human activities on the ground inside Gaza, such as waste water effluents, solid waste disposal and agricultural by-products like fertilizers, pesticides, herbicides and the like. This is strengthens that water in Gaza is now so saline and that it could be the end of agriculture there, or reduction in agricultural activity, (Larudee, 2011).

Reclaimed wastewater is an important source of irrigation in semiarid and arid zones, (Shenker et al; 2011) it have been extensively employed for various purposes around the world, including landscape irrigation, decorative fountains and landscape impoundments, crop irrigation, and groundwater recharge to overdrawn aquifers, (Xu et al., 2010). Reclaimed wastewater is an economical water resource with potential benefits in saving fertilizer, while salts was the major risk for reclaimed water irrigation. Given that there are huge potential benefits and the risks can be controlled by proper field management irrigation with reclaimed water should be encouraged and promoted, (Chen et al., 2013)

Plant growth and productivity is affected by nature's wrath in the form of various abiotic and biotic stress factors, (Mahajan & Tuteja, 2005). Salinity is one of the major environmental factors limiting plant growth and productivity in agricultural fields and one of the main component of saline soil is sodium. At high concentration, NaCl is toxic to plants because of its adverse effects on mineral and water uptake, enzyme activities, photosynthesis, protein synthesis, energy and lipid metabolism, (Nakayama et al., 2005; Parida & Das, 2005; Takahashi et al., 2009). The effects of high salinity on plants disrupts homeostasis in water potential (osmotic homeostasis) and ion distribution (ionic homeostasis). This disruption of homeostasis occurs at both

the cellular and the whole plant levels leading to death of plants and/or to a decrease in productivity, (Parida & Das, 2005; Rodríguez et al., 2005). " The earliest response is a reduction in the rate of leaf surface expansion, followed by a cessation of expansion as the stress intensifies. Growth resumes when the stress is relieved", (Parida, & Das, 2005). However, a much more extensive problem in agriculture is the accumulation of salts from irrigation water, (Taiz & Zeiger 2006). Today, ~20% of the world's cultivated land and nearly half of all irrigated lands are affected by salinity, (Zhu, 2001). Gaza Strip is also suffering from this problem where the salinity levels in most parts of the Gaza Strip are now above the WHO approved guideline of 250 mg/liter, and often much higher, (UNEP, 2009).

This problem to date is still decreasing the yield of agricultural crop, to improve the salt tolerance of plants, three approaches may be employed, (Nakayama et al., 2005). First, an increase in the biosynthesis of low molecular organic compatible solutes such as sugars, some amino acids and quaternary ammonium compounds that protect cellular molecules and adjust the turgor pressure of the cells against hyperosmotic stress, (Nakayama et al., 2005; Su & An, 2010). Second, an enhancement of the activity of different genes such as *nhx1*, *Trxh*, *Thr* and other genes. Where *nhx1* is encoded for Na^+/H^+ antiporters in plasma membrane and vacuolar membranes would maintain cytosolic Na^+ homeostasis against ionic stress by producing electrochemical H^+ gradients to the extrusion of Na^+ against its electrochemical gradient, (Blumwald et al., 2000; Nakayama et al., 2005; Yu et al., 2007). While thioredoxins (*Trxh* and *Thr*) play an important roles in the elimination of hydrogen peroxide H_2O_2, (Khan et al., 2014). Third selection based on physiological traits, improves the ability of plant to face high level of salt which could be employed for rapid and cost-effective, (Munns, R., 2002).

Mesembryanthemum crystallinum L. is a model halophytic plant native to Namibian Desert of southern Africa habitat. Under unfavorable conditions such as salinity and water stress, *M. crystallinum* L. change its mode of photosynthesis from C3 to Crassulacean acid metabolism (CAM), (Golldack & Dietz, 2001; Libik et al., 2004). CAM is a typical ecophysiological adaptation of plants to arid and saline stress. "Plants exhibiting CAM usually fix CO_2 during the night with a concomitant synthesis of malic acid, which is stored in the vacuole. During daytime the accumulated malic acid is released and decarboxylated to provide CO_2 for use via the

Calvin cycle", (Libik et al., 2004). Also, it was found many genes were expressed in high level of salinity like *myo*-inositol *O*-methyltransferase (*IMT*), *nhx1*, *McCPK1*, *Thr* and Thr*h*, compartment, (Nelson et al., 1998; Agarie et al., 2001; Golldack & Dietz, 2001; Zou et al., 2010)

Carnation (*Dianthus caryophyllus* L.) is one among the most popular commercial cut flowers of the world, native to the Mediterranean region and Central Asia, (Jawaharlal et al., 2009; Roodbaraky et al., 2012). Carnation is preferred to roses and chrysanthemums by several exporting countries, on account of its excellent keeping quality, wide range of forms, colors and ability to withstand long distance transportation, (Jawaharlal et al., 2009). Most of the agriculture production in Gaza Strip is based on cash crops for export which oriented on about 90% of the carnations (55 million carnation flower) and strawberries (2, 300 tons) produced and then marketed in the Israeli and European markets, generating sales profit, in the normal times, of $20-25 million per year, (PTC, 2009; Creti, 2010; Chetcuti & Finan, 2013). Nowadays there is close to zero export activity due to restrictions since the blockade, (FAO, 2010).

Carnation is a sensitive plant species, that need irrigation water (consume more than 1500 m^3/dunam/yr) with electrical conductivity 1.2 $dS.m^{-1}$, (Al-Najar, 2007; Haouala & Jaziri, 2009). Water as an asset, in light of Gaza's water crisis, has never been evaluated. Carnation's farm profit is nearly $530/dunam, while its water consumption is 1500 m^3/dunam/yr. That means that the Gaza Strip exports water in the form of carnations for $0.35/$m^3$.

Urban agriculture in the Gaza Strip is showing consumption of natural water resources, as if farmers are selling the country water resources in the form of carnations, strawberries with lower prices than those attached to the import price of water.

Unless eco-sanitation (re-use of treated waste water and organic wastes) is properly considered in the Gaza Strip, importing agricultural product will be the most feasible means of preserving water resources, (Al-Najar, 2007)

1.1 Objective of the study

1.1.1 General Objectives

The objective of this study is to produce new salt cultivar of *Dianthus caryophyllus* L. (omaggio) using *in vitro* selection technique and isolation of potential salinity tolerance genes from *Mesembryanthemum crystallinum* L.

1.1.2 Specific objectives

- To be able the *D. caryophyllus* L. to have an ability to cope salt stress via *in vitro* selection.
- To detect the morphological changes in selected cell lines of *D. caryophyllus* L. by coloration and ruler measurement for callus area and shoot length.
- To isolate the potential salt tolerance gene from *M. crystallinum* L. by using of RT- PCR specific sense, anti sense and non specific primers for salt tolerance gene will be used.
- To detect the salt tolerance genes by genetic analysis and later detect the similarity of salt tolerance genes with other published genes.

1.2 Significance of the study

This study will highlight the problem of increasing salinity of Gaza Strip. *D. caryophyllus* L. which is one of the floricultural crops and the most exported plant in Gaza Strip. *D. caryophyllus* L. currently consumed 1500 m^3 of agreeable water for one Donum per year which is too much due to salt status in our country.

To our knowledge this is the first study in Gaza to use *in vitro* selection techniques for enhancement of the crop quality in Gaza strip. This method is better than grafting method because this technique is limited to some plant species and it decreases the need of desalination of irrigation water, in this way this cultivar (variety) will use the reclaimed wastewater and salty water.

CHAPTER 2

LITERATURE REVIEW

LITERATURE REVIEW

2.1 Study area description

2.1.1 Geography

Gaza Strip is located at the south-eastern edge of the of the Mediterranean Sea, (Hamdan, 2012) between longitudes 34° 2" and 34° 25" east, and latitudes 31° 16" and 31° 45" north, (Aish, 2014). Its area is about 365 km^2 coastal line . It has a length of 46 km north- south and 7 to 12 km wide, (Hamdan, 2012). The climate is characterized by mild winters and dry, warm to hot summers. The average mean daily temperature ranges from 26◦C in summer to 12◦C in winter. The average annual rainfall is 335 mm per year and the average annual evaporation amounts to 1300 mm, (Ubeid, 2011).

2.1.2 Water situation

The primary water resource in Gaza is groundwater. The groundwater-bearing strata in Gaza belong to the so-called Coastal Aquifer, (Messerschmid, 2011). Groundwater aquifer is considered the main and only water supply source for all kind of human usage in the Gaza Strip (domestic, agricultural and industrial). This source has been faced a deterioration in both quality and quantity for many reasons, e.g. low rainfall, increased in the urban areas which led to a decrease in the recharge quantity of the aquifer, also increasing the population will deplete the groundwater aquifer and lead to seawater intrusion in some areas as a result in pressure differences between the groundwater elevation and sea water level, (CMWU, 2011) this encourage the relationship between sodium and chloride in the coastal area in the Gaza Strip which indicate that the aquifer experienced seawater intrusion leading to severe increase of salinity in groundwater, (Ashour, 2012). In addition to human activities on the ground inside Gaza, like waste water effluents, solid waste disposal and agricultural by-products like fertilizers, pesticides, herbicides, (Messerschmid, 2011), which made aquifer water saline, (PWA, 2012).

Agriculture is the main water consumer in the Gaza Strip with more than 70% of the total groundwater extraction, (Ashour, 2012). Many studies have demonstrated

that irrigation of treated wastewater is the most effective option for reducing the water resource deficit in Gaza, (Hamdan, 2012). The using of wastewater in agriculture have many advantages such as:

- It permits higher crop yields;
- Recycles organic matter and other nutrients to soils;
- Reduces the use of synthetic fertilizer;
- Avoids discharging pollutants to surface water bodies;
- Increases the economic efficiency of investments in wastewater disposal and irrigation;
- Conserves freshwater sources and reduces negative impacts on surface water bodies and
- Can recharge aquifers through infiltration, (Jiménez, 2006).

2.2 Carnation

Carnation *(Dianthus caryophyllus* L.) is the most famous for its use as a cut flower in the florist trade. The name carnation is derived from the Latin tern *"Carnatio"* meaning fleshiness. Caryophyllous means pink refers to the color of blooms of the original species, (Ali et al., 2008). They are found in all parts of the word but most abundant in Northern Europe and Western Asia. Carnation flowers from July to August and seed ripens from August to September, (Ilahi et al., 1995). Carnation is a member of the family Caryophyllaceae which has 93 genera, 2,395 species (Singh, 2010).

2.2.1 Rank Classification of *Dianthus caryophyllus* L.

Kingdom	Plantae- Plant
Subkingdom	Tracheobionta- Vascular plants
Superdivision	Spermatophyta- Vascular plants
Division	Magnoliophyta- Seed plants
Class	Magnoliospidia- Dicotyledons
Subclass	Caryophyllidae
Order	Caryophyllales
Family	Caryophyllaceae
Genus	*Dianthus*- pink
Species	*Dianthus caryophyllus* L.- carnation

https://plants.usda.gov/java/ClassificationServlet?source=display&classid=DICA26- 28/10/2014- 10:10pm.

2.2.1.1 Botanic description

Dianthus caryophyllus L. is a herbaceous perennial plant that can grow up to 80 cm tall, with thick, narrow, linear and succulent **leaves** and their color varies from grayish green to blue-green glaucous **leaves**, (Jaggi, 2013; Yaacob et al., 2013). The **stem** are hardy and shiny, (Jaggi, 2013). The **flowers** are sweetly scented, about 3–5 cm in size (diameter), and are either produced singly or in a bunch, (Yaacob et al., 2013); the **petals** are broad with frilled margins and the **calyx** cylindrical with **bracts** at the base, (Tah & Mamgain, 2013). The **flower** is bisexual. The **stamens** can occur in one or two whorls, in equal number or twice the number of **petals**, (Australian Government, 2005). Carnation **flowers** are naturally bright pinkish-purple in color, but other colorful cultivars of this plant had been developed such as carnations with white, red, green, and yellow flowers, (Yaacob et al., 2013). The **fruit** is in form of a capsule and contains many small seeds, (Australian Government, 2005).

2.2.1.2 Importance and uses

Carnations are excellent for cut flowers, pots, borders, edging, indoors and rockgardens, (Tarannum & Naik, 2014). Though cut carnations are traded in the world market year round.

The flower petals of carnation are candied used for flavoring fruit, fruit salads, etc. Carnations are commercially utilized for extraction of perfume in France and the Netherlands. The volatile oil of carnation contains 40% benzyl benzoate, 30% eugenol, 7% phenylethyl alcohol, 5% benzyl salicylate and 1% methyl salicylate. About 100 g of oil is obtained from 500 kg of flowers.

Carnation oils have therapeutic benefits for the treatment of skin rashes. Carnation tea or supplements have been used to relieve stress, (Jaggi, 2013).

2.2.1.3 Carnation and salt stress

There are two major constraints limit the development of Carnation (*Dianthus caryophyllus* L.) which used for cut flowers production; the availability of plant material and the quality of irrigation water. Carnation is classified as a sensitive plant species, the use of irrigation water having an electrical conductivity higher than 1.2 $dS.m^{-1}$ reduces its growth and production, (Haouala & Jaziri, 2009). This showed by

(Baas et al., 1995) who reported that high level of NaCl up to 23 mM, decreased peduncle length, reduction in growth and yield after treated with NaCl.

2.3 Salt stress

For every gram of organic matter made by the plant, around 500 g of water is absorbed by the roots, (Taiz & Zeiger, 2006). Water includes over 80% of the weight of most plant tissues so it is essential as a solvent, a transport medium for nutrients, an evaporative coolant and to provide the turgor pressure to support the growth process. There are three ways in which solutes can move from the outside solution into the root: the extra cellular (apopplastic), the intracellular (symplastic), the transmembrane pathways, (Tirajoh, 2005; Taiz & Zeiger, 2006). As water passes through the plant, salts are transported through the membranes by active or passive transport mechanisms. All salts can affect plant growth; however the most common cause of ion- specific damage in plants is Na^+, (Tirajoh, 2005).

What is stress? "Stress in physical terms is defined as mechanical force per unit area applied to an object. In response to the applied stress, an object undergoes a change in the dimension, which is also known as strain. As plants are sessile, it is tough to measure the exact force exerted by stresses and therefore in biological terms it is difficult to define stress. A biological condition, which may be stress for one plant may be optimum for another plant. The most practical definition of a biological stress is an adverse force or a condition, which inhibits the normal functioning and well being of a biological system such as plants", (Mahajan & Tuteja, 2005). Where's salt stress is a condition in which salt concentration is high enough to make the water potential in the plant excessively negative, (Etehadnia, 2009).

Under high conditions of salinity, plants face two kinds of stress: osmotic stress by reducing of absorption water from soil and ionic stress resulting from high concentrations of potentially toxic salt ions within plant cells, (Youssef, 2009).

2.3.1 Soil salinity

Soil salinity is defined as a measurement of the total amount of soluble salt in soil, (Gol, 2006). Soil salinity together with other soil physical and chemical properties,

plays an important role in plant composition, productivity and distribution due to the differences in tolerances of plant species to salinity, (Wang et al., 2007). Soil salinity occurred long before humans and agriculture, however, the problem is increasing at a rate of 10% annually, (Gol, 2006). Soil with an electrical conductivity of saturation extracts was employed to classify the soil samples into two categories, a. normal (< 4.0 ds m^{-1}) b. saline (> 4.0 ds m^{-1}) soil, (Gol, 2006; Srivastava et al., 2009). Soil salinity has been increasing due to more than one factors: low precipitation, high surface evaporation, weathering of native rocks, irrigation with saline water, entry of sea water into freshwater and poor cultural practices, (Gol, 2006).

There are three common cations associated with salinity include Na^+, Ca^{2+}, Mg^{2+}; whereas the common anions include Cl^-, SO_4^{-2}, and HCO_3^-. However, the most damaging ions are Na^+ and Cl^-, (Ali, 2010).

2.4 Halophytes versus Glycophytes

Depending on the general tolerance of salt stress, all plants can be divided into two major groups: a. halophytes, that can live on high concentrations of salt in the rhizosphere such as marine estuaries and salt marshes and grow well to withstand even 20% of salts in the soil and in most cases, successfully grow in conditions with 2-6% of salts, (Parida & Das, 2005; Rao et al., 2006) and b. non-halophytes or glycophytes, are not able to resist salts as halophytes in the same degree, (Taiz & Zeiger, 2006). Which exhibit various degrees of damage and limited growth in the presence of sodium salts, usually higher than 0.01%. However, there are great varieties in the level of salt stress tolerance within both the halophytes and non-halophytes, which include sensitive, moderately tolerant and very tolerant species. Although halophytes represent only 2% of the terrestrial plant species, they are present in about half the higher plant families and exhibit a great diversity of plant forms, (Rao et al., 2006)

One of the characteristics distinguishing halophytes from glycophytes is their ability to accumulate selectively large quantities of ions in their cells without disrupting metabolic processes, (Pessarakli, 2010). For example *Thellungiella halophila* is able to withstand dramatic salinity shock up to 500 mmol/L NaCl, (Wang et al., 2004). Where's *Atrilpex vesicaria*, a species common to salt marshes, can grow and complete its life cycle at concentration of 700 mmol/L NaCl, (Mian et al., 2011).

2.4.1 The halophyte plant *Mesembryanthemum crystallinum* L.

M. crystallinum L., also termed common ice plant, is an annual halophyte belonging to the family of Aizoaceae, order Caryophyllales (Felger et al., 2014) like *D. caryophyllus* L. Ice plant has become a model plant for studying salinity stress responses at physiological, biochemical, and gene levels, (Cosetino, 2008). Its native to southern Africa, (Felger et al., 2014).

2.4.1.1 Botanic Description

Plants spectacularly fleshy, the surfaces covered with large, watery, crystal-like vesicles. **Leaves** often 2–10 cm long, narrowed basally or with a **petiole**; blades usually obovate to broadly spatulate, or somewhat triangular. **Flowers** ca. 1 cm wide with white staminodes. **Capsules** ca. 1 cm wide, rounded like a clenched fist when dry, quickly gaping open by slit-shaped valves when wet. **Seeds** 1 mm in diameter and numerous, (Felger et al., 2014). Flowering Sep.-Feb.; fruiting Dec.- May, (Morris & Duretto, 2009). See the collection fig.2.1 below.

Fig. 2.1: ***Mesembryanthemum crystallinum*** **L.**, (Felger et al., 2014).

2.4.1.2 *M. crystallinum* L. and salt stress

The adaptation mechanisms to salinity stress in the common ice plant include metabolic transition from C3 that grown under non- stressed condition to crassulacean acid metabolism (CAM) via synthesis and cytoplasmic accumulation of osmoprotective metabolites like *myo*-inositol *O*-methyltransferase (IMT), which uses *myo*-inositol for stress-induced accumulation of a methylinositol, D-ononitol, increased the transcription of McCPK1, Thioredoxins Thr and Thr*h*, also the mechanism of tolerance includes the accumulation of sodium in the vacuolar compartment, (Nelson et al., 1998; Agarie et al., 2001; Golldack & Dietz, 2001; Zou et al., 2010).

2.5 Effects of Salt Stress on Plants

"Salt stress has threefold effects; viz. it reduces water potential and causes ion imbalance or disturbances in ion homeostasis and toxicity. This altered water status leads to initial growth reduction and limitation of plant productivity. Since salt stress involves both osmotic and ionic stress", (Parida & Das, 2005). This can be explained as high NaCl concentrations in the growth medium of plants generate primary and secondary effects that negatively affect plant growth and development. Primary effects are ionic toxicity and osmotic stress. Ionic toxicity occurs due to high concentrations of Na^+ and Cl^- in the cytoplasm of cells disturb several biochemical and physiological processes, and osmotic stress is induced by the lowering of the water potential causing turgor reduction and cellular water loss, while secondary effects of NaCl stress include inhibition of K^+ uptake, membrane dysfunction and generation of reactive oxygen species in the cells, (Jampeetong & Brix, 2009).

2.5.1 Effects of salinity on growth

High salinity causes hyperosmotic stress and ion disequilibrium that produce secondary effects or pathologies, (Yokoi et al., 2002). The initial response of salt stress is reduction in the rate of leaf surface expansion leading to cessation of expansion as salt concentration increases. Salt stress also results in a considerable decrease in the fresh and dry weights of leaves, stems, and roots, (Parida & Das, 2005). This have an agreement with (Rodr´ıguez et al., 2005) who reported that leaf area of *Asteriscus maritimus* is decreased by salinity effect.

2.5.2 Effects of salinity on water relations

Water potential and osmotic potential of plants become more negative with an increase in salinity, while turgor pressure increases with increasing salinity, (Parida & Das, 2005). The reduction in leaf area under saline stress can be considered as a avoidance mechanisms, which minimize water losses when the stomata are closed, which happens to many species under osmotic stress, (Rodr´ıguez et al., 2005).

2.5.3 Effects of salinity on leaf anatomy

The elevate level of salinity causes increases in epidermal thickness, mesophyll thickness, palisade cell length, palisade diameter, and spongy cell diameter in leaves of bean, cotton, and *Atriplex*, (Longstreth & Nobel, 1979). In other plant like sweet potato, effects including: rounding of cells, smaller intercellular spaces and a reduction in chloroplast number whereas in tomato plants: reduction of plant leaf area and stomatal density, (Parida & Das, 2005).

2.5.4 Effect of salinity on Photosynthesis

Reduction in photosynthesis of horticultural crops grown in saline environments can be attributed to decreasing of stomatal or mesophyll conductance and biochemical limitations due to Na^+ and/or Cl^- toxicities, (Paranychianakis & Chartzoulakis, 2005).

2.5.4.1 Photosynthetic pigments and proteins

Salinity stress show a decrease level in the chlorophyll and total carotenoid contents of leaves in general under salt stress. The oldest leaves start to develop chlorosis and fall with prolonged period of salt stress, (Parida & Das 2005). This has an agreement with Khavarinejad & Mostofi, 1998 who reported that in leaves of tomato, the contents of total chlorophyll (Chl-a+b) and β carotene were decreased by NaCl stress.

Also soluble protein contents of leaves decrease in response to salinity, (Parida & Das, 2005). And this decrease may have resulted from an adverse effect of NaCI on protein synthesis or proteolysis, (Khavarinejad & Mostofi, 1998).

2.6 Mechanisms of salt tolerance in plants

"The ability of plants to grow and survive under restrictive growth conditions imposed by salinity is known as salinity tolerance", (Ali, 2010). Hens plant develop biochemical and molecular mechanism to cope with salt stress, (Parida & Das, 2005).

2.6.1 Biochemical Mechanisms

Biochemical strategies include: a. Induction of antioxidative enzymes b. synthesis of compatible solutes c. Ion regulation and compartmentalization or exclusion of ions, (Parida & Das, 2005).

2.6.1.1 Induction of antioxidative enzymes

Exposure of plants to unfavorable environmental conditions such as salt stress which is a complex and imposes a water deficit causes osmotic effects on a wide variety of metabolic activities, which leads to the formation of reactive oxygen species (ROS) such as superoxide $O_2^{\bullet}$, hydrogen peroxide (H_2O_2), hydroxyl radical. These cytotoxic activated oxygen species can seriously disrupt normal metabolism through oxidative damage to lipids and to protein and nucleic acids, (Sairam & Tyagi, 2004; Parida & Das, 2005). Elimination of ROS is achieved either by antioxidant compounds such as glutathione, thioredoxin, ascorbate and carotenoids or by ROS scavenging enzymes as superoxide dismutase (SOD), catalase, glutathione peroxidases and ascorbate peroxidase (APX), (Sairam & Tyagi, 2004; Diédhiou, 2006). Superoxide radical is regularly synthesized in the chloroplast and mitochondria, (Sairam & Tyagi, 2004). Plants tolerant to ROS have evolved the capacity either to avoid the production of ROS, or increase the detoxification. For example SOD catalyses the conversion of superoxide anions to H_2O_2 and H_2O. Overexpression of this enzyme increased tolerance to abiotic stress such as salinity, (Diédhiou, 2006).

2.6.1.1.1 Thioredoxin

Thioredoxins (*Trxs*) are small proteins with molecular weight of 12-14 kDa, (Shahpiri, 2008) and occur in most organisms, from bacteria *Escherichia coli* (*E. coli*) to higher plants and animals, Trxs are thiol oxidoreductases with a pair of Cys residues that provide reducing power to a variety of stress-related enzymes, like thiol

peroxidase, and methionine sulfoxide reductase (MSR), (Zagorchev et al., 2013). In higher plants Trxs are divided into several major groups. More than 20 different *Trxs* have been identified in the genome of *Arabidopsis thaliana*, (Dolman, 2010) that can be grouped in six subfamilies. In general the *Trxs* m, f, x and y are localized in chloroplasts whereas *Trx* o is localized in the mitochondrion and *Trx* h is distributed in multiple cell compartments: cytosol, nucleus, endoplasmic reticulum as well as mitochondrion. Chloroplast Trxs are members of ferredoxin (Fd) /Trx system (Fig. 2.2), (Shahpiri, 2008). The reduction of TRX requires different reductases, depending on the cellular compartment. Cytosolic and mitochondrial TRX require compartment-specific NADPH-dependent TRX reductases (Fig.2.3), whilst plastid TRX are reduced by ferredoxin/TRX reductase, (Zagorchev et al., 2013).

Fig.2.2: Ferredoxin (Fd) /thioredoxin (Trx) system in chloroplasts,(Shahpiri, 2008).

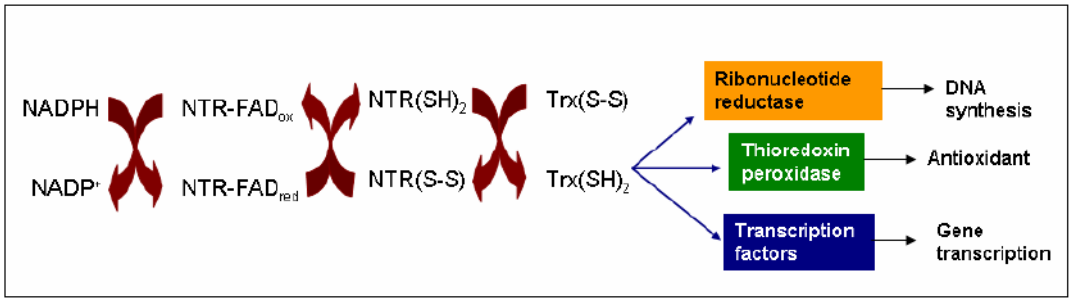

Fig. 2.3: NTR/Trx system.

Electrons flow from NADPH through FAD to the active site disulfide bond in NTR and then to the active site disulfide bond in Trx. Trx in turn acts as electron donor to Trx target proteins such as ribonucleotide reductase which reduces ribonucleotide to deoxyribonucleotide for DNA synthesis, Trx peroxidase, and transcription factors, (Shahpiri, 2008).

2.6.1.2 Synthesis of compatible solutes

To make the vacuoles have the capacity to the ionic balance, cytoplasm accumulates low-molecular-mass compounds termed compatible solutes because they do not interfere with normal biochemical reactions, so it can persevere the activity of enzymes that are in saline solutions. (Yokoi et al., 2002; Parida & Das, 2005). However, the solutes that accumulate vary with the organism and even between plant specices. These solutes classified into six major categories as:

1. simple sugars (mainly fructose and glucose),
2. sugar alcohols (glycerol and methylated inositols),
3. complex sugars (trehalose, raffinose and fructans),
4. quaternary amino acid derivatives (proline, glycine betaine, β-alanine betaine),
5. Tertiary amines (1,4,5,6-tetrahydro-2-mehyl-4-carboxyl pyrimidine),
6. sulfonium compounds (choline osulfate, dimethyl sulfonium propironate).

Several plant species naturally accumulate proline and glycine betaine as major organic osmolytes when subjected to different abiotic stresses, (Ashraf & Foolad, 2007); glycine betaine in *Amaranthus caudatus* L. (Russell et al; 1998), proline and some inorganic solutes were investigated in the tomato (*Lycopersicon esculentum* Mill. cv. Counter) leaf discs when treated with increasing NaCl concentrations. (Aziz et al., 1999).

2.6.1.3 Ion regulation and compartmentalization

The appearance of importance of ion uptake and compartmentalization are crucial not only for normal growth but also for growth under saline conditions because the stress disturbs ion homeostasis. Even though plants are glycophyte or halophyte, they cannot tolerate large amounts of salt in the cytoplasm and therefore under saline conditions they either restrict the excess salts in the vacuole or compartmentalize the ions in different tissues to facilitate their metabolic functions.

Removal of sodium from the bulk cytoplasm or compartmentalization in the vacuoles is done by a salt-inducible enzyme Na^+/H^+ antiporter, (Parida & Das, 2005); which catalyze the exchange of Na^+ for H^+ across tonoplast membranes under a proton electrochemical gradient generated by vacuolar H^+-ATPase (V-ATPase) and

vacuolar pyrophosphatase (VPPase), (Wang et Al., 2011). The V-ATPase is the dominant H^+ pump at endomembranes of most plant cells.

Under salt stress, plants keep high concentrations of K^+ and low concentrations of Na^+ in the cytosol. They do this by regulating the expression and activity of K^+ and Na^+ transporters and of H^+ pumps that generate the driving force for transport, (Parida & Das, 2005).

2.6.1.3.1 Na^+/H^+ antiporter for vacuolar Na^+ storage

Na^+/H^+ antiporters are integral transport proteins that use the proton gradient established by H^+ pumps to exchange Na^+ for H^+ across a membrane and make vacuolar compartmentalization of the cation, which are induced by saline environment, (Yokoi et al., 2002; Mansour et al., 2003; Rao et al., 2006). The first plant Na^+/H^+ antiporter gene, exhibiting high homology with yeast antiporter *NHX1*, was isolated from *A. thaliana* and designated as *AtNHX1*. Research studies of *A. thaliana* antiporters confirmed their role in salinity tolerance of plants, (Rao et al., 2006).

Na^+/H^+ antiporters have been isolated from many species like rice, tobacco cells, barley roots, red beet, reed plants and from the halophytic plants *Atriplex gmelini*, *M. crystallinum* L., (Wilson & Shannon, 1995; Wang et al., 2003; Takahashi et al., 2009; Cosentino et al., 2010).

2.6.1.3.2 Sodium efflux

Sodium efflux from root cells prevents accumulation of toxic levels of Na^+ in the cytosol and transport of Na^+ to the shoot, (Chinnusamy et al; 2005). The role of Na^+ efflux has to be considered in specific tissues in the root epidermis and around the vascular tissue, (Zhu, 2003; Maathuis, 2013). In Arabidopsis, Na^+ efflux is catalyzed by the plasma-membrane Na^+/H^+ antiporter encoded by the *SOS1* (Salt Overly Sensitive 1) gene, (Zhu, 2003).

Sodium efflux through *SOS1* under salinity is regulated by *SOS3* (a myristoylated calcium binding protein) –*SOS2* encodes a Ser/Thr protein (a serine/threonine protein kinase) complex by phosphorylation and dramatically increases Na^+/H^+ exchange activity in isolated plasma membrane vesicles, (Chinnusamy et al., 2005; Apse &

Blumwald, 2007). Among the three *SOS* loci, *SOS1* plays the greatest role in plant salt tolerance. Thus, *SOS1* may be a target for regulation by the *SOS3* and *SOS2* pathway, (Shi et al., 2000).

In addition to increasing cytosolic calcium, salt-stress induced ABA accumulation also appears to regulate the *SOS* pathway through the ABA insensitive 2 (ABI2) protein phosphatase 2C. ABI2 interacts with the protein phosphatase interaction (PPI) motif of *SOS2*. Hence, ABI2 may negatively regulate salt tolerance either by inactivating *SOS2*, or the *SOS2* regulated Na^+/H^+ antiporters such as *SOS1* or *NHX1*, (Chinnusamy et al., 2005), (Fig.2.4).

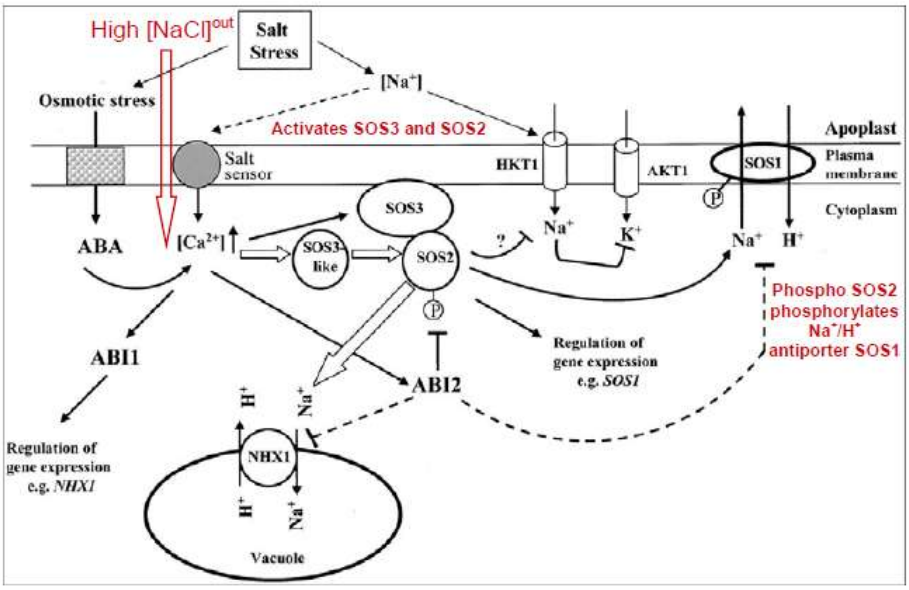

Fig. 2.4: SOS Signaling Pathway for Na^+ Homeostasis in *Arabidopsis*.

"Salt stress-elicited $Ca2^+$ signals are perceived by *SOS3*, which activates the protein kinase *SOS2*. Activated *SOS2* phosphorylates *SOS1*, a plasma membrane Na+/H+ antiporter, which then transports Na^+ out of the cytosol. The transcript level of *SOS1* is also regulated by the *SOS3-SOS2* kinase complex. *SOS2* also activates the tonoplast Na^+/H^+ antiporter that sequesters Na^+ into the vacuole. Na^+ entry into the cytosol through the Na^+ transporter *HKT1* may also be restricted by *SOS2*", (Attumi, 2007).

2.6.2 Molecular mechanism

Physiologic or metabolic adaptations to salt stress at the cellular level are the main responses to molecular analysis and have led to the identification of a large number of

genes induced by salt, (Parida & Das, 2005). However, cell biology and molecular genetics research is providing new insight into the plant response to salinity and is identifying genetic determinants that effect salt tolerance, (Yokoi et al., 2002).

Selective examples of genes/proteins induced by salt stress are given in Table 2.1. Salt tolerance is a multigenic trait which categorized into different functional groups for encoding salt-stress proteins: a. genes for photosynthetic enzymes, b. genes for synthesis of compatible solutes, c. genes for vacuolar-sequestering enzymes, and d. genes for radical-scavenging enzymes, (Parida & Das, 2005).

Tab.2.1 Selective examples of genes/proteins induced by salt stress.

Plant species	**Salt responsive genes /proteins**	**Characteristic feature(s)**
Arabidopsis thaliana	*Sal 1*	Induced by salt stress, overexpression in Arabidopsis or yeast overcomes Na^+ and Li^+ toxicity, homologous to *hal 1* of yeast
Brassica napus	*Bnd 22*	22 kDa protein, level increased by progessive or rapid water stress and salinity
Mesembryanthemum crystallinum	ppc-1 and ppc-2	Encodes PEP carboxylase, induced by salt and water stress, exogenous ABA is a poor substitute for NaCl to induce it
	Isogenes lmt 1	Encodes myo-inositol o-methyl transferase 1; induced by NaCl and osmotic stress
	Inps 1	Encodes myo-inositol 1-phosphate synthase; shows significant homology to corresponding genes in plants and yeast

(Parida & Das, 2005).

.27 Salt stress and gene regulation

Salinity, Drought and low temperature are common adverse environmental factors encountered by land plants. Knowledge of the mechanisms by which plants perceive

and transduce the stress signals is the key to understanding these responses and to genetic improvement of stress tolerance through biotechnology, (Ishitani et al., 1997).

Protein kinases are key enzymes of signal transduction and metabolic regulation, (Baur et al., 1994). Several plant receptor-Iike kinases and *MAP* kinase (Mitogen-activated protein kinases) that are induced by ABA, high salt, drought, low-temperature stress, (Hong et al., 1997; Elias, 2001).

2.7.1 Protein kinases and signal transduction pathways

Eukaryotic protein kinases make up large superfamily of homologous proteins which have two main subdivisions within the superfamily: the protein serine/threonine kinases and the protein-tyrosine kinases, (Hanks & Hunter, 1995). Plant receptor-like kinases (RLKs) are transmembrane proteins with putative amino-terminal extracellular domains and carboxyl-terminal intracellular kinase domains, with structurally resemble in domain organization to the animal receptor tyrosine kinases such as epidermal growth factor receptor, (Shiu & Bleecker, 2001). Receptor-like kinases (RLKs) play important roles in perceiving the extracellular ligands and activating the downstream pathway via phosphorylation of intracellular serine/threonine kinase domains. In *Arabidopsis*, the RLK family includes >600 members and 1100 members in rice and are classified based on their extracellular structures, (Osakabe et al., 2013). The Leucine-Rich Repeat Receptor-Like Kinase1 (LRR-RLKs) are localized at the plasma membrane and have an important roles in the transduction of plant growth, development and stress response, (Osakabe et al., 2005; Zan et al., 2013). Expression of the gene is induced by ABA, dehydration, high salt and low temperature. LRR-RLKs form a major RLK family, with more than 200 members and is classified into 15 subfamilies (LRR I–LRR XV) in *Arabidopsis*, (Osakabe et al., 2005; Osakabe et al., 2010; Osakabe et al., 2013). Other genes play an critical roles in water stress signaling like Cysteine- Rich RLK (*CRK36*), Proline-Rich- Extensin- Like RLK4 (*PERK4*) and Guard Cell Hydrogen Peroxide- Resistant1 (*GHR1*), (Osakabe et al., 2013).

Mitogen-activated protein kinase (MAPK) cascade is a universal module of signal transduction from the cell surface to the nucleus, (Ichimura et al., 2000). MAP kinases which are serine/threonine kinases, (Elias, 2001) are characterized by the sequential phosphorylation of a kinase by its upstream kinase in the order of MAPKKK-

MAPKK-MAPK, (Rai & Takabe, 2006). The MAPK cascades are known to be involved in plant environmental stress responses such as cold, freezing, heat, drought, salinity, and mechanical wounding, (Tena et al., 2001).

2.7.1.1 Calcium-Dependent Protein Kinase (CDPKs)

Calcium is a ubiquitous second messenger in eukaryotic signal transduction cascades. In plants, intracellular Ca^{2+} levels are modulated in response to environmental stimuli, including abiotic stresses such as high salinity, drought and cold, (Saijo et al., 2001; Cheng et al., 2002).

Calcium-dependent protein kinases (CDPKs) are found in a wide range of vascular and nonvascular plants as well as in green algae and certain protozoa which encoded by multigene families and have been identified in various plant species, such as Arabidopsis (*A. thaliana*), rice (*Oryza sativa*), cotton (*Gossypium hirsutum*), wheat (*Triticum aestivum*) and ice plant (*M. crystallinum*), (Zou et al., 2010).

CDPKs comprise a family of plant serine/threonine protein kinases, (Franz et al., 2011) which has four characterized domains: an N-terminal variable region, a Ser/Thr kinase catalytic domain, an autoregulatory/ autoinhibitory domain and a calmodulin-like domain. The calmodulin-like domain contains EF-hands for Ca^{2+} binding. For activation the calcium sensing domain and the kinase effectors domain are combined within one molecule, (Kong et al., 2013). At low cytosolic Ca^{2+} conc., the autoinhibitory junction domain causes the enzyme to reside in an inactive state. Rising Ca^{2+} levels as a consequence of external stress stimulus trigger a conformational change to release auto-inhibition and allow enzyme activation, (Franz et al., 2011).

2.8 Plant tissue culture and *In vitro* selection of salt-tolerant plants

Plant tissue culture is the aseptic culture of cells, tissues, organs and their components under defined physical and chemical conditions *in vitro*. Mainly developed in the early 1960s, plant tissue culture has turned into a standard procedure for modern biotechnology, and today one can recognize five major areas where *in vitro* cell cultures are currently applied: large-scale propagation of elite materials, generation of genetic modified fertile individuals, as a model system for fundamental

plant cell physiology aspects, preservation of endangered species, and metabolic engineering of fine chemicals, (Vargas & Flota, 2006).

Tissue culture techniques have been applied to the plant species in an attempt to produce new clones and cultivars with improved characteristics. In this respect, numbers of researchers have suggested that cultured tissues and cells may prove useful both in selections of the salt-tolerant plants and in studies of the physiological basis for salinity tolerance (Bekheet et al., 2006).

In recent years, tissue culture based *in vitro* selection has emerged as a feasible and cost-effective tool for developing stress-tolerant plants, (Rai et al., 2011). Plants tolerant to both the biotic and the abiotic stresses can be acquired by applying the selecting agents such as NaCl for salt tolerance, (Leva & Rinaldi, 2012; Rao & ftz, 2013). This suggests that tissue culture selection is an adequate model to select tolerant clone from overall non-tolerant populations and to research the adaptative mechanisms of plants living in saline environment, (Gandonou et al., 2006). This approach has being done using a number of plant materials (callus, suspension cultures, somatic embryos, shoot cultures, etc.) which has been screened for variation in their ability to tolerate relatively high levels of salt in the culture media, (Leva & Rinaldi, 2012).

There are two types of selection methods has 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, (Rai et al., 2011). Selection of salt-tolerant cell lines by direct selection method is considered superior compared with stepwise method of selection. (Sajid & Aftab, 2014). Because of selection of cell- lines by gradual exposure is not only responsible for the necrosis of cells but also the cause of genetic abnormalities that are usually retained by the cell population, (Nabors, 1990).

2.8.1 Callus culture

Callus refers to an undifferentiated mass of cells, (Acquaah, 2007) arranged thin-walled parenchyma cells arising from proliferating parent tissue cells. Frequently, as a result of wounding, a callus is formed at the cut end of a stem, (Doddes & Roberts, 1985) root tips onto a growth-supporting medium under sterile conditions. Under the

stimulus of endogenous growth regulators or growth regulating chemicals added to the medium, (George et al., 2008). Auxins and cytokinins both aid in the formation of most callus cells. Callus can be continuously proliferated using plant growth hormones or then directed to form organs or somatic embryos, (Stewart, 2008).

The general growth characteristics of a callus involve a complex relationship between the plant material used to initiate the callus, the composition of the medium, and the environmental conditions during the incubation period. Establishment of a callus from the explant can be divided roughly into three developmental stages: induction, cell division, and differentiation. During the initial induction phase metabolism is stimulated as the cells prepare for division. The length of this phase depends mainly on the physiological status of the explant cells as well as the cultural conditions. Subsequently, there is a phase of active cell division as the explant cells retain to a meristematic or "dedifferentiated" state. A third phase involves the appearance of cellular differentiation and the expression of certain metabolic pathways that lead to the formation of secondary products, (Doddes & Roberts, 1985). The growth rate and friability of callus produced can vary widely between explants and even within replicates of the same medium. This heterogeneity is seen in established calluses as differences in color, morphology, structure, growth, and metabolism, (Walker & Ralph, 2008), some callus growths are heavily lignified and hard in texture, whereas others break easily into small fragments. Callus may appear yellowish, white, green, or pigmented with anthocyanin, (Doddes & Roberts, 1985). Although callus has been induced for various reasons, one important application of callus is to induce somaclonal variation through which desired mutants can be selected, (Acquaah, 2007).

2.8.1.1 Somaclonal variation

Plant cells undergo different degrees of cytological and genetic changes during *in vitro* growth. Some of the changes are derived from preexisting aberrant cells in the explants used for culture. Others represent transient physiological and developmental disturbances caused by culture environments. Still others are a result of epigenetic changes, which can be relatively stable but are not transmitted to the progeny. Some variations are a result of specific genetic change or mutation and are transmitted to the

progeny. Such genetically controlled variability is known as somaclonal variation, (Stewart, 2008). The term ‘somaclone’ referred to plants derived from any form of cell culture, and to the genetic variation among such plants. The growth of plant cells *in vitro* and their regeneration into whole plants is an asexual process that involves only mitotic division of the cells. Somaclonal variation provides a valuable source of genetic variation for the improvement of crops through the selection of novel variants, which may show resistance to disease, improved quality, or higher yield, (Leva & Rinaldi, 2012). This have an agreement with (Quelros et al., 2007), who produced new cell line of potato (*Solanum tuberosum* L.) which can resist a medium containing NaCl and (Rao & Patil, 2012), produced another crop like Mung Bean (*Vigna radiata* L. Wilczek) which have an ability to resist salt in culture media.

2.8.2 Techniques of plant tissue culture

2.8.2.1 Micropropagation

Micropropagation starts with the selection of plant tissues (explant) from a health. Any part of the plant (leaf, apical meristem, bud and root) can be used as explant, (Leva & Rinaldi, 2012).

2.8.2.1.1 Micropropagation via meristem culture or axillary bud/shoot tip culture

In vitro propagation through meristem culture is the best possible means of virus elimination and produces a large numbers of plants in a short time. It is a good tool for large-scale propagation of horticultural crops including pot plants, (Rout et al., 2006). The term ‘meristem culture’ specifically means that a meristem with no leaf primordia or at most 1–2 leaf primordial which are excised and cultured, (Rout et al., 2006), also its the most reliable technique for mass multiplication since it ensures genetic stability during the regeneration process, (Scocchi et al., 2004). The term ‘meristem culture’ specifically means that a meristem with no leaf primordia or at most 1–2 leaf primordial which are excised and cultured, (Rout et al., 2006).

2.8.2.1.2 Multiplication by adventitious buds

Many ornamental and horticultural species have been successfully propagated *in vitro* by adventitious shoot initiation. New adventitious shoots can develop directly

from the explants like root, stem, petiole, leaf lamina and flower parts or indirectly from the calli obtained from these explants. Choice of explants and hormone regime to which the explants are subjected to, are two important factors in the initiation of adventitious shoots, (Jaggi, 2013).

2.8.2.2 Stages of Micropropagation

Stage 0: Preparation of donor plant

Any plant tissue can be introduced *in vitro*. To enhance the probability of success, the mother plant should be *ex vitro* cultivated under optimal conditions to minimize contamination in the *in vitro* culture, (Leva & Rinaldi, 2012).

Stage I: Initiation stage

In this stage an explant is surface sterilized and transferred into nutrient medium. Generally, the combined application of bactericide and fungicide products is suggested. The surface sterilization of explant in chemical solutions is an important step to remove contaminants with minimal damage to plant cells, (Leva & Rinaldi, 2012), then the explant should be followed by some kind of growth (e.g. growth of a shoot tip, or formation of callus), (George et al., 2008).

Stage II: Multiplication stage

The purpose of this phase is to increase the number of propagules. The number of propagules is multiplied by repeated subcultures until the desired (or planned) number of plants is attained, (Leva & Rinaldi, 2012).

Stage III: Rooting stage

Rooting shoots is a very important part of any *in vitro* propagation scheme and the success of acclimatization of a plantlet greatly depends on root system production. (George et al., 2008, Stewart, 2008). Rooting of shoots is achieved *in vitro* or *ex vitro*. *Ex vitro* (out of glass) rooting reduces the cost of production significantly. *Ex vitro* rooting is carried out by pretreating the shoots with phenols or auxins and then directly planting them in soil under high-humidity. Despite the cost factor, *in vitro* rooting is still a very common practice in many plant species because of its several advantages. Tissue culture conditions facilitate administration of auxins and other

compounds, avoid microbial degradation of applied compounds, allow addition of inorganic nutrients and carbohydrates, and enable experiments with small, simple explants (Stewart, 2008).

Stage IV: Acclimatization Stage

At this stage, the *in vitro* plants are weaned and hardened. Hardening is done gradually from high to low humidity and from low light intensity to high light intensity. The plants are then transferred to an appropriate substrate (sand, peat, compost etc.) and gradually hardened under greenhouse, (Leva & Rinaldi, 2012).

2.8.3 Regeneration methods of Plants in culture

2.8.3.1 Organogenesis

Organogenesis is the production of organs: either shoot or root. Organogenesis *in vitro* depends on the ratio of auxin and cytokinin and the ability of the tissue to respond to phytohormones during culture. Organogenesis takes place in three phases. In the first phase the cells become competent; next, they dedifferentiate. In the third phase, morphogenesis proceeds independently of the exogenous phytohormone. Organogenesis *in vitro* can be of two types: direct and indirect, (Stewart, 2008).

2.8.3.1.1 Indirect organogenesis

Indirect organogenesis is refer to the formation of organs indirectly via a callus phase. Induction of plants using this technique does not ensure clonal fidelity, but it could be an ideal system for selecting somaclonal variants of desired characters and also for mass multiplication. Induction of plants via a callus phase has been used for the production of transgenic plants in which:

- the callus is transformed and plants regenerated or
- the initial explant is transformed and callus and then shoots are developed from the explant, (Stewart, 2008).

2.8.3.1.2 Direct organogenesis

Adventitious shoots which arise directly from the tissues of the explant without within callus stage can provide a reliable method for micropropagation. However, the

induction of direct shoot regeneration depends on the nature of the plant organ from which the explant was derived and is highly dependent on plant genotype.

Adventitious shoots can be formed *in vitro* on pieces of tissue derived from various organs (e.g. leaves, stems, flower petals or roots); in others species, they occur on only a limited range of tissues such as bulb scales, seed embryos or seedling tissues, (George et al., 2008).

2.8.3.2 Somatic Embryogenesis

Somatic embryogenesis is a process whereby somatic cells differentiate into somatic embryos. Somatic embryos resemble zygotic embryos morphologically, (George et al., 2008), which are bipolar structures, arise from individual cells, (Sharma & Agrawal, 2012). Somatic embryogenesis may occur directly or via a callus phase. Direct somatic embryogenesis is preferred for clonal propagation as there is less chance of introducing variation via somaclonal mutation. Indirect somatic embryogenesis is sometimes used in the selection of desired somaclonal variants and for the production of transgenic plants. One advantage of somatic embryogenesis is that somatic embryos can be directly germinated into viable plants without organogensis; thus it mimics the natural germination process, (Stewart, 2008).

2.8.4 Factors Affecting Plant Tissue Culture

2.8.4.1 Choice of explant

The tissue which is obtained from the plant to culture is called an explant, (Idowu et al., 2009). The part of the plant (the stock plant or mother plant) from which explants are obtained, depends on:

- the kind of culture to be initiated;
- the purpose of the proposed culture;
- the plant species to be used.

2.8.4.2 The cultural environment

Physical requirements such as light temperature, photoperiod and relative humidity can influence the process of *in vitro* differentiation from various explant tissue including the meristem and shoot tip explants, (Vasil & Throp, 1994;

Khachatourians et al., 2002). Culture response can be affected by duration of exposure to light and its intensity and quality, (Khachatourians et al., 2002).

High humidity in the culture room should be avoided because it increases contamination, (Smith, 2013).

2.8.4.3 Surface Sterilization

The first condition for the success of a culture is asepsis. The maintenance of aseptic (free from all microorganisms) or sterile conditions is essential for successful tissue culture procedures. To maintain an aseptic environment, all culture vessels, media and instruments used in handling tissues, as well as explant itself must be sterilized. The importance is to keep the air, surface and floor free of dust. All operations should be carried out in laminar airflow sterile cabinet.

Sterilization is the process of making explants contamination free before establishment of cultures. Various sterilization agents are used to decontaminate the tissues. These sterilants are also toxic to the plant tissues, hence proper concentration of sterilants, duration of exposing the explant to the various sterilants, the sequences of using these sterilants has to be standardized to minimize explant injury and achieve better survival, (Badoni & Chauhan, 2010). The disinfectants usually used are sodium hypochlorite, calcium hypochlorite, ethanol, mercuric chloride, hydrogen peroxide and silver nitrate. Most laboratories use sodium or calcium hypochlorite or various commercial bleaches for surface sterilization of explants. Since these sterilizing agents are toxic to the plant tissue, contamination must be removed without killing the plant cells, (Mihaljević et al., 2013).

Choice of treatment is usually determined by the nature of the explant and the extent of external contamination. A wetting agent is usually included (e.g , a drop of household detergent or Tween) to improve contact with the sterilant, (Hall, 1999).

2.8.4.4 Culture Media composition

Plant material will only grow *in vitro* when provided with specialized media (George et al., 2008). The basic composition of a culture medium includes the following: macro- and microelement, vitamins, plant growth substance and carbon source. The macro, microelement and vitamin constituents have remained fairly

constant, although different formulations have been developed over the years. The Murashige and Skoog medium has been one of the most commonly used culture media since its inception. Prior to that, medium formulation devised by White and Heller were widely used. Many of the medium formulation such as Erikson, B5, Scheck and Hildebrandt have evolved from the MS medium composition. Ono or more of these medium types are often tried in preliminary experiments to assess the efficiency of each, (Khachatourians et al., 2002).

2.8.4.5 Plant growth regulators

Growth regulators are organic compounds, other than nutrients, which in small amounts promote, inhibit or modify any physiological process in plants, (CASPP, 1954). Synthetic chemicals with similar physiological activities to plant growth substances, or compounds are usually termed plant growth regulators, (George et al., 2008). They are generally classified into the following groups; auxins, cytokinins, gibberellins and abscisic acid, (Leva & Rinaldi, 2012)

In fact, the pioneering work of Skoog and Miller implicating the interaction between auxins and cytokinins in the initiation of roots and shoots has made possible the *in vitro* regeneration of a wide array of plant species. It is now common knowledge that the concentrations and ratios of these two classes of compounds influence callus production, organogenesis and somatic embryogenesis. For callus induction high levels of auxins are usualy required, whilst shoot production generally requires higher levels of cytokinins, (Khachatourians et al., 2002). The common auxins used in plant tissue culture media include: indole-3- acetic acid (IAA), indole-3- butric acide (IBA), 2,4-dichlorophenoxy-acetic acid (2,4-D) and naphthalene-acetic acid (NAA). The commonly cytokinins used in culture media are BAP (6-benzyloaminopurine), 2iP (6- dimethylaminopurine), kinetin (N-2-furanylmethyl-1H-purine-6-amine), Zeatin (6-4- hydroxy-3-methyl-trans-2-butenylaminopurine) and TDZ (thiazuron-N-phenyl-N-1,2,3 thiadiazol-5ylurea), (Leva & Rinaldi, 2012).

2.8.4.6 Vitamins

Vitamins are needed by plant cells as essential intermediates or metabolic catalysts. Unlike animals, are able to produce their own requirements. Cultured plant

cells and tissues can however become deficient in some factors; growth and survival is then improved by their addition to the culture medium, (George et al; 2008). The vitamins commonly used are thiamine (vitamin B1), pyridoxine (B6), nicotinic acid (niacin), and thiamine. Other vitamins such as biotin, folic acid, ascorbic acid (vitamin C), and vitamin E (tocopherol) are sometimes added to media formulations. Myoinositol is added to most plant culture media to improve the growth of cultures, (Stewart, 2008).

The concentrations of each can vary significantly between the different media and the nature of the plant type, (Hall, 1999; George et al., 2008).

2.8.4.7 Medium pH

The pH of the medium is important since it influences the uptake of various components of the medium as well as regulating a wide range of biochemical reactions occurring in plant tissue cultures, (Stewart, 2008). A pH higher than 6 gives a fairly hard medium and a pH below 5 does not allow satisfactory gelling. Thus, the effective range of pH for tissue culture medium is limited, (Bahita & Ashwath, 2005) The optimal pH for carnation callus induction by Nontaswatsri and Fukai was 5.75, (Wang, 2006). Whereas (Ali et al., 2008) maintained pH 5.71±0.5. for carnation shoots cultures. (Ilahi et al., 1995) adjusted the pH 5.7 for shoots and roots initiation.

2.8.4.8 Carbon source

Most plant-cell cultures are non autotrophic and are therefore entirely dependent on an external source of carbon, (Hall, 1999) In most cases sucrose has been one of most commonly used in tissue cultures. Glucose has also been used to some extent. in recent years studies have demonstrated that maltose is a very effective carbohydrate source for improved response of plant tissues in culture, (Khachatourians et al., 2002).

2.9 Pervious study

- **Thioredoxin**

Zhang et al., 2011, demonstrated that *OsTRXh1* is induced by salt and abscisic acid treatments in rice. They also analyzed the levels of hydrogen peroxide produced in transgenic plants and the results shown that more hydrogen peroxide is produced in the extracellular space of *OsTRXh1* knockdown plants than in wild-type plants, whilest the *OsTRXh1* overexpression plants produce less hydrogen peroxide under salt stress. These results shown that *OsTRXh1* regulates the redox state of the apoplast and influences plant development and stress responses.

Bandehagh et al., 2013, reported that under high salt stress there were more than 419 proteins detected in root samples of rapeseed (*Brassica napus L.*), one of these proteins *Trxh, (thioredoxin h*) which act as a major defense system against oxidative damage by reducing the disulphide bonds of oxidized proteins. In this experiment Trxh-1 presented only in salinity levels, with higher expression in Hyola308 that exposed to 350 mM NaCl. Such a pattern of variations suggested that, accumulation of Trxh-1in under salinity treatment has a protective role in tolerant genotype.

- **Selection and adaptation:**

Tam & Lang, 2003, observed that genetic variability was applied by *in vitro* selection in rice. The researchers cultured seeds of 12 varieties of rice on various media for callus induction. They found that the four types of rice genotypes *Doc Do, Pokkali, Trang Thai Lan* and *KDM105* were suitable for embryogenic callus formation. Then they transferred parts of the calli to NaCl to create genetic variability in rice cell culture to obtain salt tolerance plants through *in vitro* selection. They found that both treated and non–treated calli that survived in 1.0 and 1.5% NaCl and these benefit for further studies which should be made to regenerate plants from these materials.

Bekheet et al., 2006, in their study they initiated onion callus from aseptic seedlings which was exposed to different levels of salt mixture. For selection under salt stress, regenerated shoot buds derived from tolerant callus cultures were exposed to the different levels of salts mixture. The best results of salt tolerance ratio were

reached at 2000 ppm salts. The tolerant shoot buds were *in vitro* rooted and successfully adapted to free-living conditions.

Gandonou et al., 2006, demonstrated that *in vitro* selection techniques can be used to generate salt-tolerant cell lines in sugarcane and to study physiological and biochemical indicators of salinity tolerance in this plant. Thus salt tolerance seemed to be related to the efficiency of a tissue to modulate the level of inorganic and organic solutes in response to salt stress. These results indicate that Cl^- exclusion combined to K^+, proline and soluble sugars accumulation are the main option to counteract the negative effects of salt stress in sugarcane selected calli.

Zinnah et al., 2013, were aimed to obtain salt tolerant genotype of *BRRI Dhan* 38 and *Chini Kanai (local variety)* rice varieties through somaclonal variation. They were used different concentration and combinations of growth regulators supplemented to MS medium for induction the callus stage which then transferred to the best regeneration medium at different concentrations of NaCl, such as, 0, 50, 100, 150 and 200 mM, for salt stress responses to check the inherent capacity of calli to regenerate on medium under salt stress condition. There results showed that *Chini Kanai* is more salt tolerant then *BRRI Dhan* 38 and this is due to somaclonal variation.

CHAPTER 3

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals, kits and disposables

Chemicals, kits and disposables used in this study are shown in Table 3.1 and primers sequences in Table 3.2.

Tab. 3.1 Chemicals, kits and disposables used in this study.

Item	Company
RNeasy Plant minikit (50)	Qiagen, Germany
QIAGEN OneStep RT-PCR Kit	
DNA master mix	Promega, USA
wizard sv gel and PCR clean-up kit	
100bp DNA Ladder (250µL)	
Blue orang 6x Promega	
Ethidium bromide	
Agarose	
Tris- Base	
Primers	Metabion, Germany
Phytagel	Sigma, USA
Kanamycin	Wako Reagent, Japan
Hygromycin	Himedia, India
TDZ Thidiazuron	
NAA α-Naphthaleneacetic acid	
BA 6- benzylaminopurine	
Sodium hypochlorite	
Sucrose	
Sodium chloride	
Murashige and skoog medium	
Phyta jar	
Sterile bottle	
Petri dishes	
Sterile loop	
Serological pipette 25ml	
Tips yellow	
Tips blue	
Tips neutral	
Storage box (5-10 µL)	
Storage box (200 µL)	

Freezing box	
Rack for multiple uses	
PCR centrifuge tube	
PCR racks	
Parafilm	
Glass lenses	
Detachable face shield `	
Sterile tips (10-200 µL)	Labcon, USA
1 ml filter tips, sterile	
Chemical mask	Al- Ghsain, Gaza
Liquid nitrogen	

Tab. 3.2 Primers used to amplify.

Primer	Sequence	Tm	MW	Length in bp
PF1	5'- ATG GCG TTT GAT TTG AGT AA-3'	68	8963	20
PR1	5'- TAT GTA CTC TCT GTC GAA TGG TT-3'	69	9795	23

The primers sequences were obtained from a previously published work, (Cosentino, 2008).

3.1.2 Instruments and Equipments

The major instruments and equipments used in this study are listed in Table 3.3.

Tab.3.3 The instruments and equipments used in this study.

Instruments/ Equipments	Purpose to use in the research	Company
Safety cabinet	For genetically and tissue culture used	N-Biotek, Inc
Vortex	For genetically used specially for (mixing)	LW, scientific, Inc-Geargea
Microcentrifuge	For genetically used	BioRad, Germany
Thermal cycler	For amplification used	Biometra
Gel electrophoresis apparatus	For separation and analysis of macromolecules (DNA) and their fragments, based on their size and charge.	Clever Scientific Limited, UK
Electrophoresis power supply	Charging the gel electrophoresis instrument	Parklaam 36, Belgium
NanoDrop spectrophotometer	For detection the concentration of mRNA, PCR product	Implen, Germany
UV Transilluminator Documentary system	For documentation of gel and plates	Clever Scientific Limited, UK
Microwave	In the gel preparation, media	JAC, Egypt
Hot plate	For stock minerals (stirrer,	Scilogex, USA

	heating), media preparation (stirrer, heating)	
PH meter	To adjust PH (media)	Selecta, Spain
Autoclave	To sterile media, equipments (tips, forceps,----etc)	Cristofoli, Brazilian
Heat block	For genetically used (incubation)	Boeco, Germany
Refrigerator 4ºC	To keep the nutrients, media of tissue culture	Selecta, Spain
Refrigerator -20ºC	To keep DNA, restriction enzymes, PCR kits and other samples	ORSO and Selecta, Spain
Refrigerator -80ºC	For storage RNA samples	Selecta, Spain
Plant Growth chamber	For tissue culture	Human lab. Instrument co. Koria
Analytical balance	To weight nutrients, minerals	Adam, UK
Pipettes 5, 10, 50, 200, and 1000 µL	For transport a measured volume of liquid	Labmed, Boeco

All aqueous buffers, solutions and media were prepared with double distilled water unless other wise indicated;

Buffer for biological grade.

All buffers, solutions and media components are outlined in Appendix I.

3.1.3 *Mesembryanthemum crystallinum* L. source

Seeds and aerial parts of the ice plant (*M. crystallinum* L.) were collected from the costal region of Gaza. The aerial parts were identified based on the written description and image comparison based on Madi, 2001 book.

Seeds were planted in the same soil of coastal site in pots. Seeds plant was exposed to 12 h day- night light cycle photoradiation.

At the age of 4 to 5 weeks the plants were stressed by irrigation with 400 mM NaCl over two weeks in order to induce Crassulacean acid metabolism (CAM) metabolism by salt stress.

3.1.4 *Dianthaus caryophyllus* L. (omaggio) Carnation source

Seedling for carnation *D. caryophyllus* L. (omagio) variety were obtained from Palestinian Agricultural Relief (PAR) who obtained them from Holland. The omaggio variety belongs to Selecta company, this variety is characterized as excellent quality flower, big head, round petals and intense color. This variety is an open to modification.

The seedling of carnation was grown in the reticular and plastic house in the roof of Biology and Biotechnology lab. at the Islamic University of Gaza.

3.2 Methods

3.2.1 Setting of the study

The study was performed at the Biology and Biotechnology department at Islamic University of Gaza.

3.2.2 Isolation of Salinity tolerance gene

Total RNA was isolated from *M. crystallinum* L. leaves. In brief, 100 mg frozen plant materials were ground to a fine powder under liquid nitrogen using a mortar and pestle. The powder was then transferred into 2 ml Eppendorf tube and the extraction steps completed using RNeasy Plant mini- kit reagent as follows:

1. The liquid nitrogen was allowed to evaporate, without making the tissue thawing.
2. Buffer RLT (450 µl) was added and vortexed vigorously.
3. The lysate was then transferred into a QIAshredder spin column (provided), placed in a 2 ml collection tube and centrifuged for 2 min at full speed. Carefully the supernatant of the flow-through was transferred to a new microcentrifuge tube without disturbing the cell-debris pellet in the collection tube.
4. Half volume of ethanol (96–100%) was added to the cleared lysate and mixed immediately by pipetting up and down for several times.
5. The sample was transferred to an RNeasy spin column (provided) including any precipitate that may have formed and placed in a 2 ml collection tube. The lid was closed gently, and centrifuged for 15 s at (≥10,000 rpm). The flow-through was discarded and the collection tube was reused in the next step.
6. Washing buffer RW1 (700 µl) was added to the RNeasy spin column. Then the lid was closed gently and centrifuged for 15 s at (≥10,000 rpm) to wash the spin column membrane. The flow-through was discarded and the collection tube was used in the next step.

7. Washing buffer RPE (500 µl) was added to the RNeasy spin column, the lid was closed gently and centrifuged for 15 s at (≥10,000 rpm) to wash the column. The flow through was discarded and the collection tube was reused in the next step.
8. Again buffer RPE (500 µl) was added to the RNeasy spin column, the lid was closed gently and centrifuged for 2 min at (≥10,000 rpm) to wash the column. The flow through was discarded and the collection tube was reused in the next.
9. The RNeasy spin column was placed in a new 2 ml collection tube and the old collection tube was discard with the flow-through. The lid was closed gently, and centrifuged at full speed for 1 min.
10. The RNeasy spin column was placed in a new 1.5 ml collection tube. (30–50 µl) RNase-free water was added directly to the spin column membrane. The lid was then closed gently, and centrifuged for 1 min at (≥10,000 rpm) to elute the RNA.
11. Total RNA was quantified spectrophotometrically.
12. Finally, the RNA was stored at -80^{o}C until RT- PCR analysis.

3.2.3 cDNA synthesis and PCR amplification

cDNA synthesis and PCR were performed using a One Step RT-PCR Kit.
A number of trials were carried out for this purpose:

- Specific primers were used for cDNA synthesis and amplification;
- Specific sense primer was used in the amplification with and without oligo DT primer that was used for reverse transcription and amplification.

The cDNA synthesis and amplification reaction from 84 ng/ µl total RNA sample was performed in the reaction steps as summarized below:

1. The kit components (dNTP Mix, 5x QIAGEN OneStep RT-PCR Buffer and RNase-free water), also the template RNA and primer solutions were allowed to thaw on ice.
2. The reaction was prepared according to table 3.4.

Tab. 3.4 First RT-PCR reaction mixture.

Component	Volume (µL.)		Final conc.
	Trial 1	Trial 2	
RNase-free water (provided)	9	9	-
5x QIAGEN OneStep RT-PCR Buffer	5	5	1X
dNTP Mix (containing 10 mM of each dNTP)	1	1	0.4 mM
Primer F1 (10 µM)	1	1	0.4 µM
Primer R1 (10 µM)	1	-	0.4 µM
Oligo DT primer (10 µM)	-	1	0.4 µM
QIAGEN OneStep RT-PCR Enzyme Mix	1	1	
Template RNA	7	7	
Total	25	25	

3.The thermal cycler was programmed as in table 3.5.

Tab. 3.5 Temperature cycling program for first RT-PCR reaction.

	Time	Temperature
Reverse transcription	30 min	50°C
Initial PCR activation step	15 min	95°C
3-step (annealing temp.)		
15 cycles	1 sec 1 sec 1.5 min	94°C 48°C 72°C
20 cycles	1 sec 1 sec 1.5 min	94°C 50°C 72°C
10 cycles	1 sec 1 sec 1.5 min	94°C 52°C 72°C
Final externship	10 min	72°C

The annealing temperature of the reaction was set low in order to lower the stringency of the reaction and allow for partially matched targets to amplify.

The RT product was stored at -20 to -24.6ºC.

The amplified fragments were run on 1.2% agarose gel prepared in Tris acetate ethyldimethyl tetra acetic acid (TAE) buffer (Appendix 1) and visualized by UV

illuminator after staining with ethidium bromide. Blue orang 6x dye (Promega, USA) was used as loading/tracking dye. DNA bands were compared with 100 bp ladder as to estimate the size of amplified fragment of required band. The amplified products were purified and then used for a second PCR with specific sense (F1), oligo- DT primer and sense anti sense primer (F1 and R1). The reaction was prepared according to table 3.6.

Tab. 3.6 For DNA production reaction mixture for 50µl.

Component	**Volume (µl)**		**Final conc.**
	Trial 1	**Trial 2**	
5x Go tag polymerase (ready mix)	25	25	1X
PF1 (10 µM)	1	1	0.4 µM
PR1 (10 µM)	1		0.4 µM
Oligo- DT (10 µM)		1	0.4 µM
DNA template	2	2	0.4 µM
Nuclease-free water	21	21	
Total	50	50	

The thermal cycler was programmed as in table 3.7.

Tab. 3.7 Temperature cycling program for PCR production

	Time	**Temperature**
Initially denatured	5 min	95 °C
20 cycles	30 sec. 30 sec. 1.5 min	95 °C 52 °C 72 °C
Final extension	10 min	72 °C

The amplified products were run again on 1.2% agarose gel prepared in TAE buffer (Appendix 1) and visualized by UV illuminator after staining with ethidium bromide.

3.2.3.1 Purification of amplified fragment from gel PCR products

The amplified PCR product was gel purified using wizard sv gel and PCR clean-up kit (Promega, USA) as follows:

First: Dissolving the Gel Slice (product of F1& R1)

1. After loaded the samples and visualized by UV illuminator, the required bands were cut.
2. The bands of insert were put in weighted a 1.5ml microcentrifuge tube for each DNA fragment to be isolated and the weight recorded again.
3. Membrane Binding Solution was added at a ratio of 10µl of solution per 10mg of agarose gel slice.
4. The mixture was mixed by vortex and incubated at 50–65°C for 10 minutes or until the gel slice was completely dissolved. The tube was vortexed every few minutes.

Second: DNA Purification by Centrifugation

1. The SV Minicolumn (provided) was placed in a Collection Tube for each dissolved gel slice or PCR amplification.
2. The dissolved gel mixture was transferred to the SV Minicolumn assembly and incubated for 1 minute at room temperature.
3. The SV Minicolumn was centrifuged in a microcentrifuge at (12,000rpm) for 1 minute. The SV Minicolumn was removed from the Spin Column assembly and the liquid in the Collection Tube discarded. The SV Minicolumn was then returned to the Collection Tube.
4. Membrane Wash Solution (700µl) was added to the column after being diluted with 95% ethanol, the SV Minicolumn was centrifuged for 1 minute at (12,000rpm). The Collection Tube was discarded and placed the SV Minicolumn back in the Collection Tube.
5. Step 4 was repeated with 500µl of Membrane Wash Solution and centrifuged the SV Minicolumn assembly for 5 minutes at (12,000rpm).
6. The SV Minicolumn was removed from the centrifuge, the Collection Tube was discarded and recentrifuged for 1 minute with the microcentrifuge.
7. The SV Minicolumn was transferred to a clean 1.5ml microcentrifuge tube.
8. 50µl of Nuclease-Free Water were added directly to the center of the column without touching the membrane with the pipette tip then incubated at room temperature for 1 minute and centrifuged for 1 minute at (12,000rpm).
9. The SV Minicolumn was discarded and the microcentrifuge tube containing the eluted DNA stored at –20°C to -24.5°C.

3.2.4 DNA sequencing and sequence analysis

DNA sequencing was performed on an automated sequencer (*San Diageo*). Sequences were analyzed using *DNASIS* software, and databank searches were conducted through the *BLAST* program.

3.3 Tissue culture (selection method)

3.3.1 Sterilization of plant tissue culture tools

Plant tissue culture tools were sterilized three times, first steam sterilization was before working in the aseptic condition (hood), second 3% H_2O_2 and third heat sterilization were used during the working in the aseptic condition (hood).

3.3.2 Growth condition of *Dianthaus caryophyllus* L. (omaggio)

1. Carnation genotype (Omaggio), 5- 8 cm shoots, were taken from green-house mother carnation plants.
2. Leaves were removed carefully from the nodes.
3. Shoots were surface sterilized for 15 min in sodium hypochlorite (1% available chloride) and one drop of Tween-20 and then rinsed twice for 10 min each in sterile water.
4. The first three nodes below the shoot meristem were cut into 1-mm thick explants, each containing a node and leaf scar.
5. All *in vitro* cultures were incubated at 25°C under a 16-h photoperiod at 2664 to 3500 lux provided by cool white fluorescent lamps.

3.3.2.1 *In vitro* sodium chloride treatments

Explants grown on Medium I were directly exposed to different concentration of NaCl (0, 50, 150 and 200 mM) for 4 weeks for initiation a shock selection calli see Appendix II medium Ia and medium Ib .

3.3.2.2 Regeneration of Shoots

For plant regeneration, the part of calli was cut into small pieces. Calli were then inoculated on regeneration media with different combination and concentrations of

hormones, supplemented with different conc. of NaCl (0, 50, 150 and 200 mM) see Appendix II medium IIa, IIb and III.

3.3.2.3 Rooting and hardening

The regenerated shoots were subcultured onto medium IV (Appendix 2).

After the size of carnation developed (~ 2.0 to 5.0 cm) well rooted plants of culturing were hardened in growth chamber. For hardening industrial soil was used by washing the roots with sterile distil water followed by transfer to soil in pots. The pots were covered with polyethylene bags to keep humidity level high, which had holes in bags to help the plants acclimatized.

3.4 Statistical Analysis

The data were analyzed using Paired- Sample T test (SPSS Version 14) for callus induction. Variance analysis (two- way ANOVA) was applied to the data at shooting stage to analyze the data for significant differences. The values were also compared for significant difference using LSD test.

CHAPTER 4

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

4.1 Isolation of salinity tolerance genes

4.1.1 Reverse transcriptase polymerase chain reaction (RT-PCR)

Full length complementary DNA (cDNA) was synthesized from total RNA extracted from *Mesembryanthemum crystallinum* L. leaves and amplified with specific primers (F1, R1), (F1, oligo- dT) under low stringency condition (as explained in the materials and methods section). The amplified products (~1420 bp and ~1500 bp) were visualized by UV illuminator after staining with ethidium bromide on a 1.2% agarose gels (Fig.4.1). The amplified products were purified by wizard sv gel and PCR clean-up kit. Later, a second PCR was made using specific sense (F1), oligo- dT primer and sense anti sense primer for (F1 and R1), (Fig. 4.2). This step was done in order to confirm and increase the concentration of the products (as illustrated in the materials and methods section).

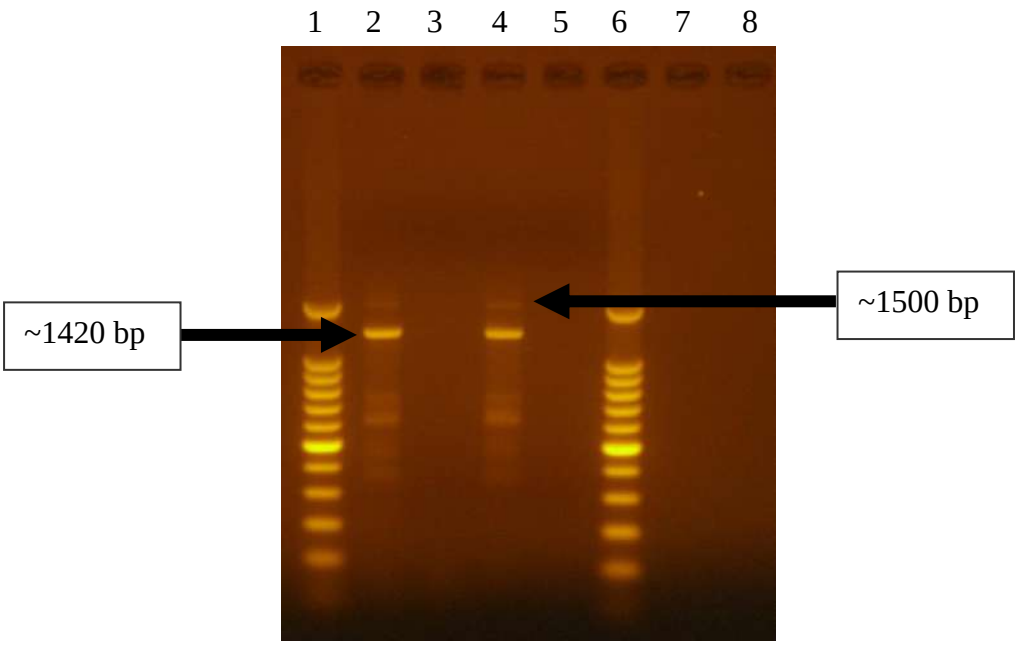

Fig. 4.1: Ethedium bromide stained gel for first RT-PCR.
Lane 1, 6 100 bp DNA ladder; Lane 2, 4 (F1, oligo-dT) shows ~1420 and ~1500 bp and lane 3, 5 shows a negative water control.

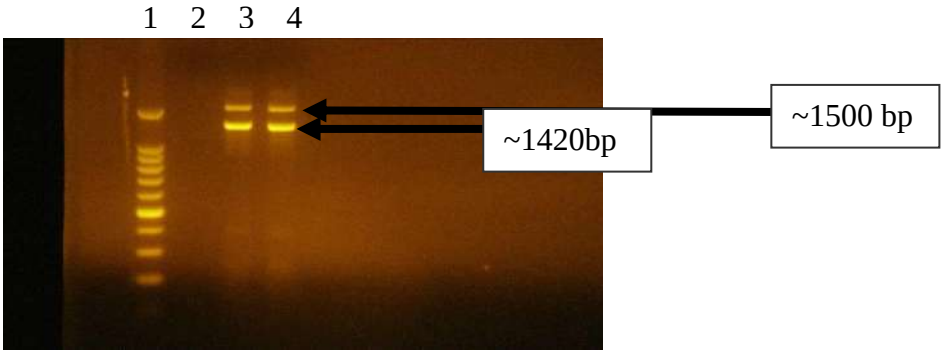

Fig. 4.2: Ethedium bromide stained gel for PCR production.
Lane 1 100 bp DNA ladder. Lane 3 (F1, R1), 4 (F1, oligo-dT) shows ~1420 and ~1500 bp and lane 3 shows a negative water control.

During this work, there was an attempt to isolate salinity tolerance genes from the plant *M. crystallinum* L., which was obtained from the wild, based on previously published primers sequences originally designed for amplification of the gene for vacuolar Na^+/H^+ antiporter (Gene bank accession number AM746985.1) (Cosentino, 2008). Unfortunately, in this study there is no results obtained at first attempt, maybe because of genetic variation of the wild plant. This variation may occur due to environmental changes or geographic distribution and reflects local adaptation to different heterogeneous environments. Developmental variation was reported to exists within many species, which likely reflects adaptations to different natural environments including abiotic signals such as light, temperature, wind, humidity, water availability or nutrient resources as well as most biotic components of the surrounding environment from pathogens to competitors (Blanco et al., 2005). Moreover, genetic diversity could result from different landscapes (Jogaitė et al., 2006). Consequently, a modification of the RT-PCR condition by lowering the annealing temperature and using the generic oligo-dT primer for cDNA synthesis in combination with the specific primers (figure 4.1 and 4.2). In this work, the potential salinity tolerance genes were proposed to be ubiquitous because of saline growth conditions and will be preferentially amplified in such low stringency amplification conditions. The use of oligo-dt primer for conversion of mRNA into cDNA through reverse transcription was effective in enriching for nonspecific cDNA synthesis, while although not completely specific the other primers served for PCR amplification in a one-step RT-PCR .

4.1.2 DNA sequencing

The amplification fragments indicated in Figure 4.2 were sliced from the gel, purified and submitted for sequence determination. The obtained sequences were analyzed by blast search of the National Center for Biotechnology Information (NCBI) Blast server (http://www.ncbi.nlm.nih.gov/BLAST/). The blast search revealed a putative protein kinase gene (GenBank ccession number AK229991, 1341 bp length) with 88% identity to the first sequence of isolate. As the approximate size of band submitted for sequencing was larger, the obtained sequences were reanalyzed once again using the Plant Functional Genomics Research Group database specialized in Arabidopsis resource sequences (http://rarge.gsc.riken.jp/). Surprisingly, the *Arabidopsis thaliana* At5g06690 mRNA for putative thioredoxin gave a 100% match with 1426 bp length, equivalent to our isolated gene. The identified gene confer the plant a character for salt tolerance. Similarly, the second isolated amplicon (larger band size, figure 4.2) showed 95% identity to the *Beta vulgaris* subsp. *vulgaris* serine/threonine-protein kinase EDR1-like under (GenBank accession number XM-010679950) by (http://www.ncbi.nlm.nih.gov/BLAST/). The later gene is also implicated in response to salinity stress.

Salt treatment of *M. crystallinum*, was previously demonstrated to activate many genes to cope with salt stress, (Yang & Yen, 2002). Agarie et al., 2001 demonstrated that a number of genes are activated in response to salt stress including thioredoxin H, thioredoxin, vacuolar H^+-ATPase, Myo-inositol 4-O-methyltransferas and serine/threonine protein kinase-like protein. These genes and others are upregulated and are mainly involved in stress responses, defense-related, ion homeostasis, protein folding and degradation, signal transduction and transport facilitation. In addition, 7% of the induced transcripts were functionally unknown, including several novel genes. Therefore, the two isolated fragments in this study may be similarly implicated in coping with salt stress (thioredoxin) and in disease resistance (serine/threonine-protein kinase EDR1-like). Xiong et al., 2003 reported that plants which are constantly exposed to biotic (i.e., pathogen infection and insect herbivory) and abiotic (i.e., high or low temperature, drought, and salinity) stresses, survive these challenges, by developing elaborate mechanisms to perceive external signals and to manifest adaptive responses with proper physiological and morphological changes.

Therefore, our isolated genes could be introduced into plants in order to genetically modify them to tolerate high salinity irrigation water and increase their economic value.

4.2 Tissue culture (selection method)

4.2.1 Sterilization of plant tissue culture tools

During this study, 3% hydrogen peroxide H_2O_2 was used after steam sterilization of tissue cultures tools. This technique with steam sterilization of the tools and heat sterilization of the tools ensured that contamination by bacteria or fungi did not appear on the culture under *in vitro* or *ex vitro* conditions. This method on the other hand was not followed in other plant tissue cultures studies. While previously published that preventing the cell culture contamination is a dream, (Ryan, 2008), during this study the contamination were not appeared.

This technique was performed based on the pervious medical study for sterilization the tools of medical equipments, (Patel, 2003).

The uses of this sterilization agent was based on its principle of works by producing destructive hydroxyl free radicals which can attack membrane lipid, DNA and other essential cell components, (Rutala et al., 2008).

4.2.2 Callus induction and regeneration under stress and non-stress condition

During this study, activated charcoal was used to ensure that browning could not appear on the cells (based on the earlier studies of plant culturing and the problems of browning which damages the tissues). However it was observed that the best callus production was obtained using media I which is devoid of charcoal. This may be because activated charcoal (AC) absorbs the nutrient from the media and delays the growth of cells. This observation agreed with some researches finding the actual quantity of plant growth regulators concentration available to the plant tissues is not known exactly, also it is not clear what are all the chemicals released by AC, that are naturally present in it, (Thomas, 2008).

During the present work, tolerant cell lines of carnation were obtained by direct selection process by directly placing the plant nods on the selection media without

prior induction of the calli in controlling media. This approach was previously followed by others (Khorami & Safarnejad, 2011) who aimed at *in vitro* selection of *Foeniculum vulgare* for salt tolerance by placing the root, hypocotyle and cotyledon of explants on Murashige and Skoog (MS) medium supplemented with 0, 50, 100 and 150 mM of NaCl. While this process of selection was different from other studies which induced the calli in controlling medium before transferring the cells into selection medium, this techniques were shown in Gandonou et al., 2006 study who develop sugarcane (*Saccharum* sp.) calli on MS medium supplemented with 68 mM NaCl by direct process. Rao & Patil, 2012 also develop mung bean (*Vigna radiata* L. Wilczek) callus by sudden exposure to high conc. of NaCl. Sajid & Aftab, 2014, who produced salt- tolerant callus of two potato cultivars (Cardinal and Desiree) (*Solanum tuberosum* L.) by sub- culturing the calli on MS medium containing different concentrations of NaCl (0, 20, 40, 60, 80, 100, 120 or 140 mM). This approach on the other hand was followed by few other studies, which induced the calli in controlling medium before transferring the cells into selection medium.

Also, this study may be consider the first study which depended on the external dimension to estimate the growth of callus, while other studies was used the length of shoots or roots, leaf area to determine the growth of plants as in Shahid et al., 2011 and Riaz et al., 2013 reporting. Other study was determined the growth based on the diameter of roots as in Shafi et al., 2010 demonstrations. Thus, in this study the growth determination for every stages was depended on the scale measurement. These methods were used for many reasons:

- This study is one of the first study in Islamic University- Gaza, which used plant growth chamber instrument and this device have many troubleshooting;
- The troubleshooting of the plant growth chamber which continued to many months constricted the study. This made any other methods for estimation the growth not available, also the physiological tests.

In this study, several morphological and coloration changes were observed with the addition of salt in the medium over successive subcultures as shown in table 4.1 and fig. 4.3- 4.7.

A decrease in cell lines (callus proliferation) and coloration was observed with increasing the salt conc. Thus, these results confirm earlier findings of Sajid & Aftab, 2014, who reported changes in potato callus morphology from off-white to blackish-brown at 120 mM.

Tab.4.1 Coloration of carnation callus cultures (omagio) at different NaCl levels. (0-200 mM) supplemented to MS medium under light conditions at day 10, 20 and 30.

Salt con. mM	**Color of callus during experimental period 30 days**		
	10 days	**20 days**	**30 days**
0	++++ Green	**++++** Green	+++Green
50	**++++** Green	**++++**Greenish-yellow	++Greenish-yellow with some whitish portion
100	**+++** Greenish-yellow	++Greenish-yellow with some whitish portion	+Yellowish-brown,
150	++Yellowish to brownish green	++Yellowish-brown	++Yellowish-brown
200	**+** Yellowish to brownish green	+Yellowish-brown	+Yellowish-brown

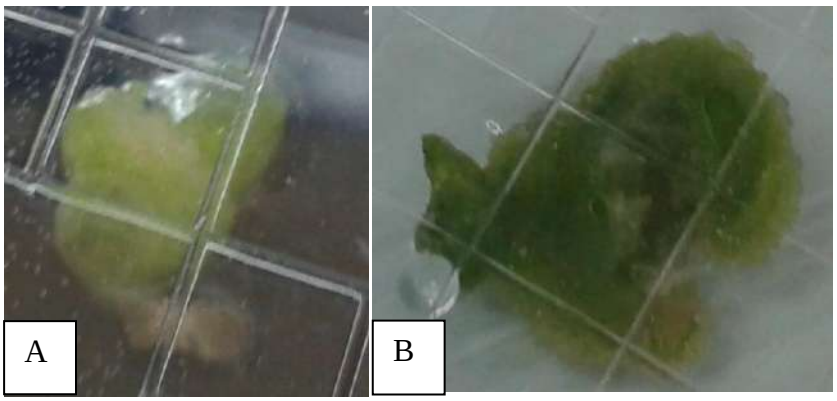

Fig. 4.3: Callus production A (0 mM NaCl) on medium I .
A; Callus production on MS medium supplemented with charcoal, B; Callus production on MA medium without AC.

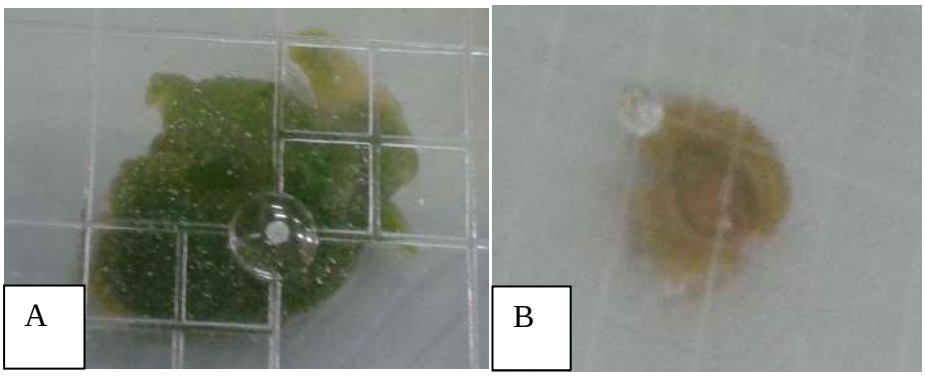

Fig. 4.4: Callus production B1 (50mM NaCl) on medium I.
A; live cells (selected), B; dead cells.

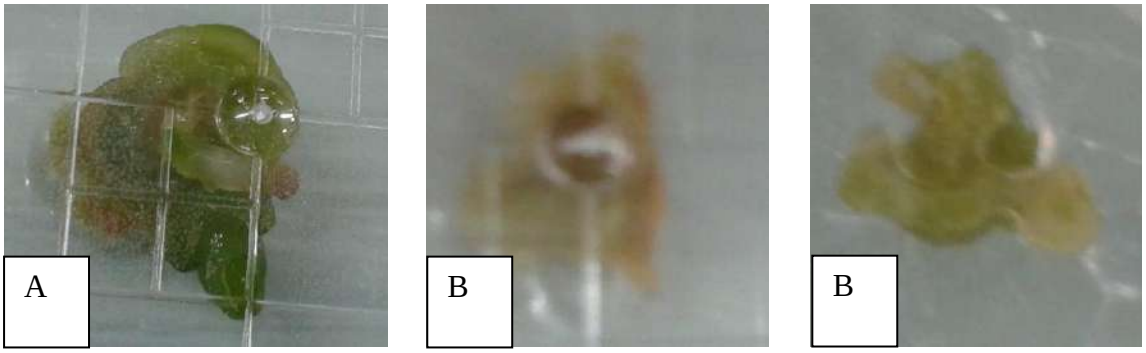

Fig. 4.5: Callus production B2 (100 mM NaCl) on medium I.
A; live cells (selected), B; dead cells.

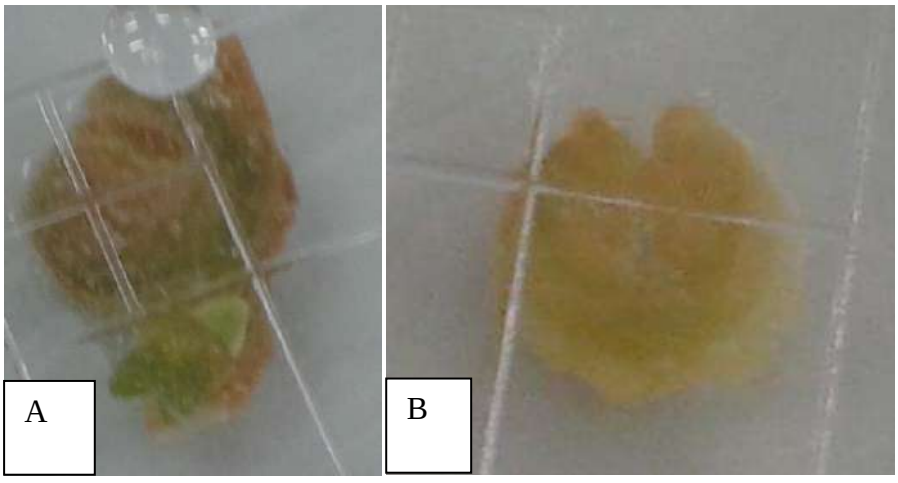

Fig. 4.6: Callus production B3 (150 mM NaCl) on medium I.
A; 25% live cells (selected) based on the image shown, B; dead cells.

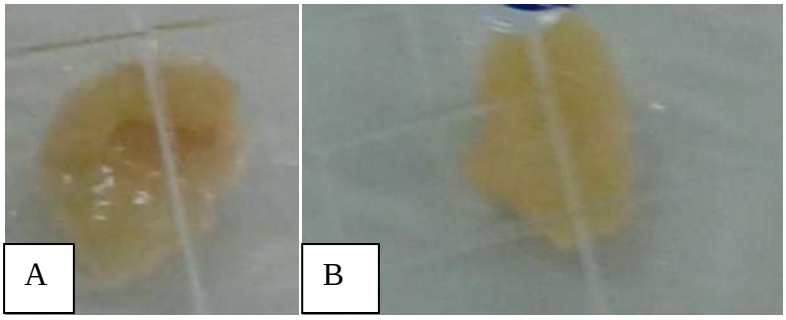

Fig. 4.7: Callus production B4 (200 mM NaCl) on medium I.
A and B represented dead cells.

The Result from this study indicate that callus under photoperiod condition undergoes cellular differentiation for shoot formation. This is in an agreement with Wright & Northcote, 1973 who reported that many callus tissues have been found to have the ability to undergo cellular differentiation. In some cases, this occurs spontaneously, whereas in others the differentiation can be experimentally induced, usually by an appropriate supply of auxins and cytokinins.

4.2.2.1 Salinity effect on callus induction

A significant difference in callus induction was found at different conc. of NaCl (0- 150 mM), whereas no significant difference was observed in callus induction at 200 mM NaCl. The data obtained by using Paired-Sample T test presented in table 4.2 showed that salt treatments significantly affected the induction of callus ($P<0.05$).

Tab. 4.2 Salinity effect on callus

		Mean Difference	Std. Deviation	Relative Growth Rate	Sig. (2-tailed)
A	0 mM at zero time - 0 mM after 20 days	-.43167	.15329	71.54%	.001
B1	50 mM at zero time - 50 mM after 20 days	-.13167	.05742	27.11%	.003
B2	100 mM at zero time - 100 mM after 20 days	-.10500	.08408	20.65%	.028
B3	150 mM at zero time - 150 mM after 20 days	-.03833	.03656	6.99%	.050
B4	200 mM at zero time - 200 mM after 20 days	-.006667	.01033	1.44%	.175

Data represents average of six replicates of 5 treatments. The mean difference is significant at the .05 level. The relative growth rate of callus was calculated as the (ELf - ELi)/ELi where ELf and ELi were the final and the initial external length, respectively.

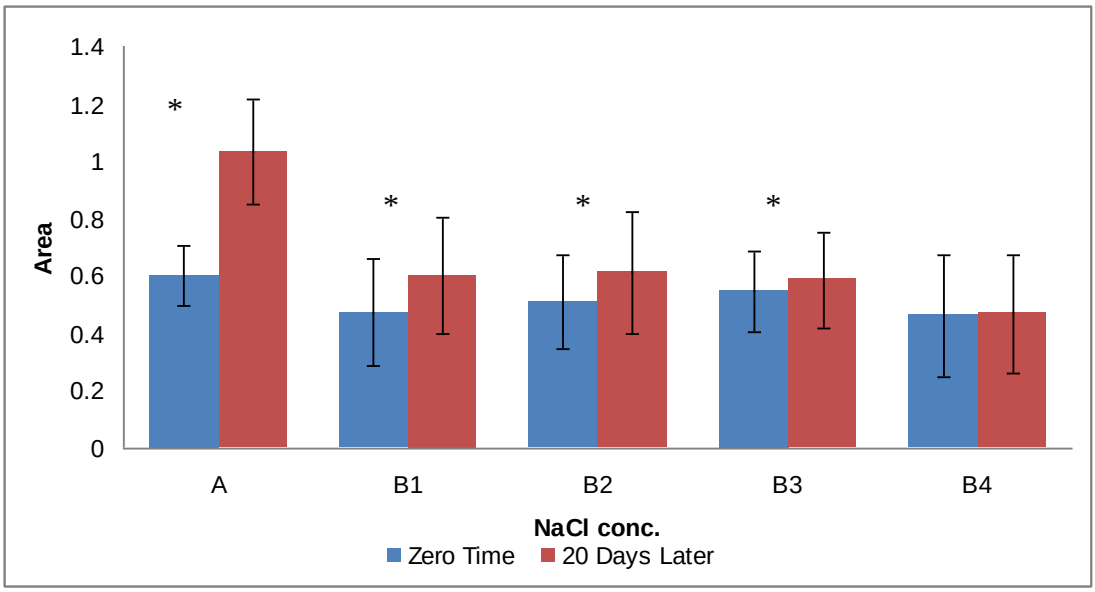

Figure 4.8: Callus induction at different conc. of NaCl.
(A control 0, B1 50, B2 100, B3 150, B4 200 mM NaCl). The Area was calculated and blotted in X axes by measuring and multiplying the calli longest and shortest dimensions in cm.
Symbol * indicate to the significant results.

In general, the relative growth rate (Tab. 4.2) indicate that there are no selected cells developed but B1 and B2 have some cells (green color and morphology) similar to A and these cells made a difference in the growth and later used for shooting stage. The development of these cells corroborate the theory of somaclonal variation which arises by environmental culture and the genetics variation of plant cells to overcome these environmental stress. This have an agreement with Anwar et al., 2010 whose study was concerned in the somaclonal variation and their finding among the *in vitro* regenerated plants show the potential of callus culture for creating genotypic variability and subsequent selection for desirable traits in sweet potato. Also, our results supported the earlier findings by Akhtar et al., 2012 who cultured calli of twenty wheat varities on MS medium under four salinity levels *i.e.*, 0, 50, 100 and 150 mM NaCl to develop salt tolerant wheat somaclones and there results indicated that somaclones developed having salt tolerance at different salinity levels can be shown in marginally saline lands. Other demonstrating by Khan et al., 2014 whose study concern in the somaclonal variation through *in vitro* plants selection and cultured of potato on MS media containing various salt concentrations such as 0, 25, 50, 75 and 100 mM NaCl. Thus there results were promise about somaclonal variation as useful tool to develop salt tolerance of potato tubers.

Also, the results indicated that there were a decreases in callus induction at high level of NaCl, this confirms other results by Khaleda et al., 2007 who studied the effect of different conc. of NaCl (0.1%- 0.3% w/v) and there results shown that the highest dose of NaCl 0.3% w/v inhibited callus induction compared to 0.1% and 0.2% NaCl in the MS media for Rice (*Oryza sativa* L.)

4.2.3 Plant regeneration from callus culture of carnation

In an attempt to study the regeneration potential of callus cultures of carnation, proliferated calli (30-days-old) were shifted to the regeneration media. The effect of different growth regulators (BA alone and NAA + BA) was tested on the regeneration potential of carnation callus cultures with a combination of different NaCl conc. (0, 50, 100 mM).

BA was found to be the best hormone in selected callus line. The effects of BA on organogenesis and shoot differentiation have been reported by Ali et al., 2008, whilst NAA and BA show less effect in shoot formation may be due to the presence of active site for roots formation on nods. To avoid these results in this study, it concern on the uses of BA alone for shoot formation and multiplication, see the collection figure 4.10. These have an agreement with Guse & Larsen, 2001 who reported that nodes contain actively dividing cells where root formation is likely to occur most readily. Also, Pessarakli, 2001 wrote in his book that roots sometimes develop even without callus from the nodes.

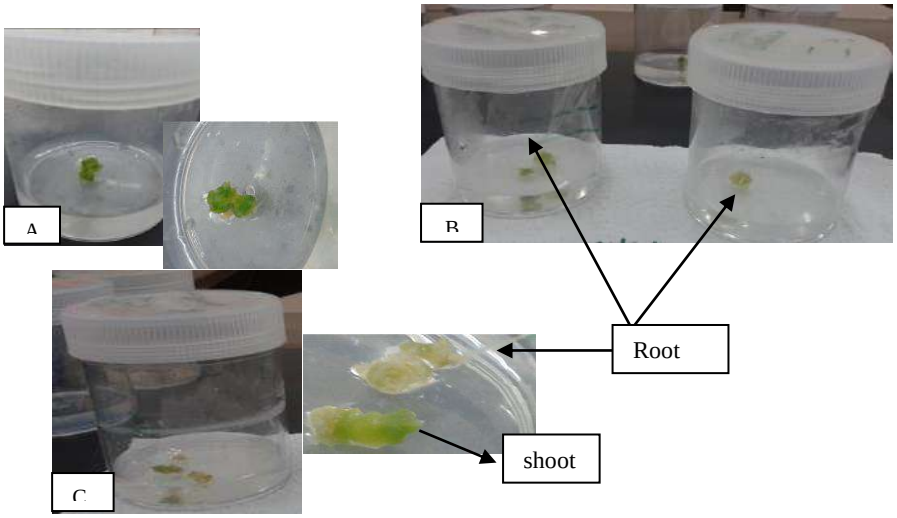

Fig. 4.9: Shooting stage from callus.
A; Shooting stage from callus A to A.
B; Shooting stage from callus A to A*.
C; Shooting stage from callus B2 to B2*.
Note: * meaning that medium for shooting have a combination of NAA with BA.

4.2.3.1 Salinity effect on shooting growth

Salt treatments significantly (P<0.05) affected the growth rate of shoots (Tab. 4.3, 4.4). The data which obtained from two- way ANOVA indicated that there were no significant difference for the time and the significant was for the conc. of NaCl. Consequently Post hoc tests (LSD) was used to assay the sig. differences between groups of selection and the control. All data were significant except the difference between 50 mM and 100 mM NaCl due to the difference in length of shooting between the two groups were very small.

Tab.4.3 Salinity and time effect on shooting

Source	F	Sig.
Time	2.043	.125
NaCl	4.926	.013
Time * NaCl	.555	.763

The mean difference is significant at below 0.05 level.
Depended variable: length.

Tab. 4.4 Salinity effect on shooting

I		J	Mean difference (I-J)	Sig.
A	**0 mM NaCl**	50 mM NaCl	1.5083*	.004
		100 mM NaCl	1.0333*	.042
B1	**50 mM NaCl**	0 mM NaCl	-1.5083*	.004
		100 mM NaCl	-0.4750	.341
B2	**100 mM NaCl**	0 mM NaCl	-1.0333*	.042
		50 mM NaCl	0.47500	.341

Data represents average of 3 replicates for each treatment where every treatments have 4 replicates. (No. of treatments 3).
(*) The mean difference is significant at below 0.05 level.

Decrease in regeneration frequency of NaCl-selected callus line may be due to the difficulties of regeneration from salt media or somaclonal vrariation, see the figures from 4.10- 4.12. The results from this study have matched other finding like Zinnah et al., 2013 who obtained salt tolerant genotype of BRRI Dhan 38 and Chini Kanai (local variety) rice varieties via somaclonal variation. There results indicated that plant regeneration of BRRI dhan 38 was 80% at 0 mM NaCl, but decreased to 20% at 50 mM NaCl, while there was 0% plant regeneration at 150 mM NaCl for BRRI 38.

On the other hand Chini Kanai plant regeneration on the no-stress medium was 60% and at 150 mM it decreased to 20% and there was no regeneration at 200 mM NaCl. This confirms that Chini Kanai is more salt tolerant than BRRI Dhan 38. Similarly Zahid et al., 2014 reported that callus induction and plant regeneration percentage decreased with the increase of salt doses.

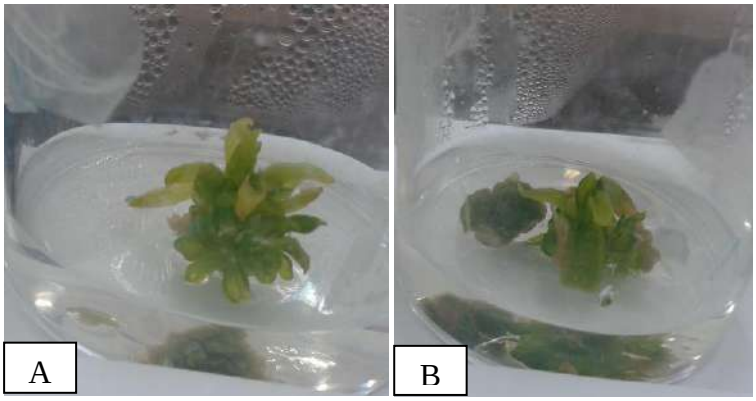

Fig. 4.10: Shooting stage A (0 mM NaCl) on medium II, a .
A& B Shooting stage from callus A to A.

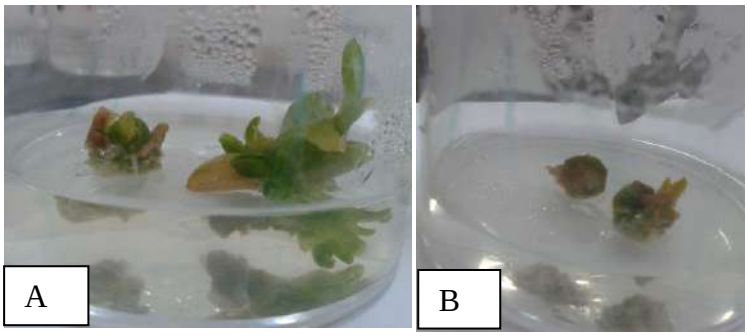

Fig. 4.11: Shooting stage B1 (50 mM NaCl) on medium II, a.
A; Shooting stage from callus B1 to B1 and B; shooting stage from callus B1 to B1 (dead cells).

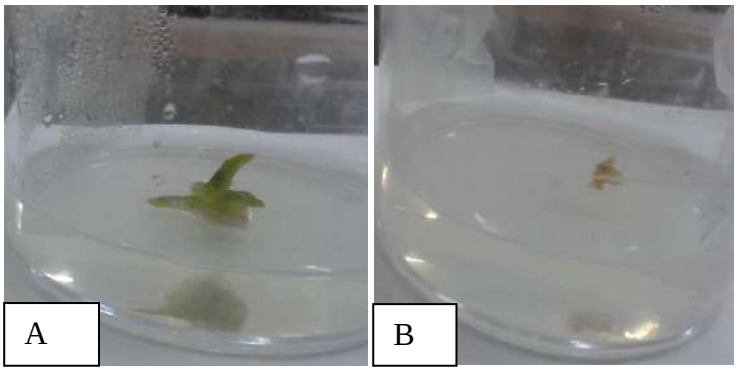

Fig. 4.12: Shooting stage B2 (100 mM NaCl) on medium II, a.
A; Shooting stage from callus B2 to B2 and B; shooting stage from callus B2 to B2 (dead cells)

4.2.4 Rooting of vegetative area

In this study the roots observed in the MS media after 10 days of incubation and it have an agreement with Ali et al., 2008.

The effect of NAA was tested for formation potential of carnation root cultures with a combination of different NaCl conc. (0, 50, 100 mM).

It was found a decrease in the number, length and thickness of roots with increase in the conc. of NaCl, this results was logical due to the root systems were the direct injured part caused by salinity, so the current study have an agreement with Bahmani et al., 2012 who worked on the *in vitro* response of MM.106 apple rootstock to increasing concentrations of NaCl (0, 20, 40, 80, 100 and 120 mM). They found at 20 mM NaCl the shoot length, fresh weight and root length was increased significantly as compared with the control, while at high concentrations of salt (120 mM NaCl) the proliferation and root formation were decreased in length. Pandey & Chikara, 2014 also, studied *in vitro* condition incubation *Stevia Rebaudiana* 'Bertoni' under salinity and drought stress on growth, where they subjected plants to different conc. of NaCl and mannitol, they found a significant decrease in shoot number, shoot length, root number and root length with the increasing concentration of NaCl and mannitol (25, 50, 75 and 100mM).

Notes:

- The number of roots were not detected due to long incubation period (poor security situation in Gaza).
- Figures 4.13- 4.15 were taken after the security situation in Gaza was improved, (age of plant 120 days).

Fig. 4.13: Multiple shooting and rooting of shooting area for A(0 mM NaCl) group, (120 days).

Fig. 4.14: Multiple shooting and rooting of shooting area for B1 (50 mM NaCl) group, (120 days).

Fig. 4.15: Multiple shooting and rooting of shooting area for B2 (100 mM NaCl) group, (120 days).

4.2.5 Hardening and Acclimatization of Carnation

Hardening of carnation plants was successfully accomplished for carnation cultivar (omagio) for A and B2 groups, while acclimatized carnation to the glasshouse conditions was not accomplished happened due to the troubleshooting in growth chamber (humidity in the instrument) in the last days in hardening under growth

chamber condition. The plants established under *ex-vitro* conditions were also healthy (Fig. 4.16- 4.18).

Fig. 4.16: Carnation grown under *ex vitro* condition group A (0 mM NaCl).

Fig. 4.17: Carnation grown under *ex vitro* condition group B1 (50 mM NaCl).

Fig. 4.18: Carnation grown under *ex vitro* condition group B2 (100 mM NaCl).

CHAPTER 5

CONCLUSION AND RECOMMENDATION

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

In this study, two genes (putative thioredoxin and serine/threonine-protein kinase EDR1) were isolated and found to be potentially involved in salinity tolerance. These two genes represent a potential means for genetically conferring plants a salinity tolerance character.

Furthermore, this study is the first to use *in vitro* selection techniques which could be an important means of improving salt tolerance in plants. Salinity tolerant cell lines were successfully created by direct selection process.

The different levels of growth of cells in high salt-media, suggests the occurrence of genetic variability of cells.

Healthy carnation were successfully regenerated from calli maintained under different conc. of NaCl. The promising morphological features of the regenerated carnation plants maintained at high conc. of NaCl (100 mM) with 10 dS/m EC in *in vitro* and *ex vitro* conditions encourage further research in this field.

5.2 Recommendation

The results of this study recommended many aspects in the future work such as;

- Examination of a sudden shock of high levels of salts on cells to get the tolerate cells is a good suggestion;
- Application of further research in this field with testing the biochemical, physiological and morphological changes during salt stress;
- Expand the work to the fields to test the stability of salt tolerance of carnation;
- Investigation of somaclonal variation and its impact on salt tolerance at the molecular level;
- Testing the genetic stability of selected carnation is recommended; Genes from this study must be tested and used later use to enhance the tolerance of carnation or other ornamental and crops which are glycophytes;

- Use the genetic modifications to enhance the tolerance of carnation to cope the salts.
- Using of H_2O_2 during the sterilization of plant tissue culture tools is recommended.

CHAPTER 6

REFFERENCES

REFFERENCES

- Acquaah, G. (2007). Principles of Plant Genetics and Breeding. First Edition, Blackwell Publishing Ltd. P. No. 1, 38, 54, 55.
- Agarie, S., Cushman, M., Kore-eda, S., Deyholos, M., Galbraith, D & Cushman, J. (2001). Gene Expression Profiling of Salinity Stress Responses Using Expressed Sequence Tag (EST)- Based Microarrays in the Common Ice Plant, *Mesembryanthemum crystallinum*. *Plant and cell physiology*. 42 (Supplement), s130.
- Aish, A. (2014). Estimation of Water Balance Components in the Gaza Strip with GIS Based WetSpass Model. *Civil and Environmental Research*. 6: 77-85.
- Akhtar, S., Niaz, M., Rahman, S., Younas, M & Zaffar, M. (2012). Somaclonal Variation for Development of Salt Tolerance in Selected Wheat (*Triticum aestivum*) Cultivars. *Int. J. Agric. Biol.*14: 600- 604.
- Ali, A. (2010). Exploring the Possibility of Transforming Food Crops for Salinity Tolerance using the TMT Gene Encoding Thiol Methyltransferase Enzyme. Master Thesis. University of Waterloo. Canada. P. No. 103.
- Ali, A., Afrasiab, H., Naz, S., Rauf, M & Iqbal, J. (2008). An Efficient Protocol for *In Vitro* Propagation of Carnation (*Dianthus caryophyllus*). *Pak. J. Bot*. 40: 111- 121.
- Al-Najar, H. (2007). Urban agriculture and Eco-sanitation: The Strategic Potential Toward Poverty Alleviation in the Gaza Strip. *RICS Research paper series*.7: 1- 28.
- Anwar, N., Kikuchi, A & Watanabe, K. (2010). Assessment of Somaclonal Variation for Salinity Tolerance in Sweet Potato Regenerated Plants. *African Journal of Biotechnology*. 9: 256-7265.
- Aps, M & Blumwald, E. (2007). Minireview Na^+ Transport in Plants. *FEBS Letters*. 581: 2247–2254.

- Ashour, E. (2012). Modeling of Orchards Irrigation Demand Under Vulnerable Climate Change and the Sequencing Effect of Soil Salinization in Gaza Strip. Master Thesis. Islamic University- Gaza. Palestine. P. No. 1.
- Ashraf, A & Foolad, M.R. (2007). Roles of Glycine Betaine and Proline in Improving Plant Abiotic Stress Resistance. *Environmental and Experimental Botany*. 59: 206–216.
- Attumi, A. (2007). A Study of Salt Tolerance in *Arabidopsis thaliana* and *Hordeum vulgare*. Doctor Thesis. University of Glasgow, U.K. P. No. 22.
- Australian Government, (2005). The Biology and Ecology of *Dianthus caryophyllus* L. (Carnation). Office of the Gene Technology Regulator.
- Aziz, A., Tanguy, J.M & Larher, F. (1999). Salt Stress-Induced Proline Accumulation and Changes in Tyramine and Polyamine Levels are Linked to Ionic Adjustment in Tomato Leaf Discs. *Plant Science*. 145: 83- 91.
- Baas, R., . Nijssen, H., Berg, T & Warmenhoven, M. (1995). Yield and Quality of Carnation (*Dianthus caryophyllus* L.) and Gerbera (*Gerbera jamesonii* L.) in a closed Nutrient System as Affected by Sodium Chloride *Scientia Horticulturae*. 61: 273- 284.
- Badoni, A & Chauhan, J. (2010). *In Vitro* Sterilization Protocol for Micropropagation of *Solanumtuberosum* cv. 'Kufri Himalini'. *Academia Arena*. 2: 24- 27.
- Bahita, P & Ashwath, N. (2005). Effect of Medium pH on Shoot Regeneration from the Cotyledonary Explants of Tomato. Biotechnology. 4: 7- 10.
- Bahmani, R., Gholami, M., Mozafari, A & Alivaisi, R. (2012). Effects of Salinity on *In vitro* Shoot Proliferation and Rooting of Apple Rootstock MM.106. *World Applied Sciences Journal*. 17: 292- 295.
- Bandehagh, A., Uliaie, E & Salekdeh, G. (2013). Proteomic Analysis of Rapeseed (*Brassica napus L.*) Seedling Roots Under Salt Stress. *Annals of Biological Research*. 4: 212- 221.
- Baur, B., Fischer, K., Winter, K & Dietz, K. (1994). cDNA Sequence of a Protein Kinase from the lnducible Crassulacean Acid Metabolism Plant *Mesembryanthemum crystallinum* L.,Encoding a SNF-1 Homolog. *Plant Physiol*. 106: 1225-1226.

- Bekheet, S, Taha, H & Solliman, M. (2006). Salt Tolerance in Tissue Culture of Onion (*Allium cepa* L.). *Arab J. Biotech.* 9: 467-476.
- Blanco, C., Vigo, B & Koornneef, M. (2005). From Phenotypic to Molecular Polymorphisms Involved in Naturally Occurring Variation of Plant Development. *Int. J. Dev. Biol*. 49: 717-732.
- Blumwald, E., Aharon, G & Apse, M. (2000). Sodium Transport in Plant Cells. *Biochimica et Biophysica Acta*. 1465: 140- 151.
- Chen, W., Lu, S., Jiao, W., Wang, M & Chang, A. (2013). Reclaimed Water: A safe Irrigation Water Source?, *Elsevier B.V*. 8: 74- 83.
- Cheng, S., Willmann, M., Chen, H & Sheen, J. (2002). Calcium Signaling through Protein Kinases. The Arabidopsis Calcium-Dependent Protein Kinase Gene Family. *Plant Physiology*. 129: 469- 485.
- Chetcuti , P & Finan, L. (2013). Gaza Blockade: Help them Grow. ACF International. Paris.
- Chinnusamy, V., Jagendorf, A & Zhu, J. (2005). Understanding and Improving Salt Tolerance in Plants. *Crop Science Society of America*. 45: 437-448.
- Coastal Municipalities Water Utility (CMWU). (2011). Annual Report on Water Status in the Gaza Strip. Palestine.
- Committee of the American Society of Plant Physiologists (CASPP). (1954). Nomenclature of chemical plant regulators. American Society of Plant Biologists.
- Cosentino, C. (2008). Na^+ /H^+ Transporters of the Halophyte *Mesembryanthemum crystallinum* L. Ph. D hesis. Darmstadt. Italia. P. No. 20.
- Cosentino, C., Schliebs, F., Bertl, A., Thiel, G & Homann, U. (2010). Na^+/H^+ Antiporters are Differentially Regulated in Response to NaCl Stress in Leaves and Roots of *Mesembryanthemum crystallinum*. *New Phytologist*. 186: 669–680.
- Creti, P. (2010). Market Analysis of Three Economic Sectors in the Gaza strip The Food Processing, Dairy and ICT Sectors. Oxfam. U.K.
- Diédhiou, C. (2006). Mechanisms of Salt Tolerance: Sodium, Chloride and Potassium Homeostasis in Two Rice Lines with Different Tolerance to Salinity Stress. Ph. D Thesis. University of Bielefeld. Germany. P. No. 8.

- Doddes, J & Roberts, L. (1985). Experiments in Plant Tissue Culture, Second Edition, Press Syndicate of the University of Cambridge. United Sate of America. P. No. 54, 55.
- Dolman, F. (2010). Functional Characterization of Plant Cytosolic Thiroedoxins. Ph. D Thesis. University of Adelaide, Wait Campus. Australia. P No. 20.
- Elias, D. (2001). Characterization of ESI47, A salt Stress Regulated Protein Kinase from *Lophopynrrn elongatwn*. Master Thesis. Concordia University. Canada. P. No. 12, 13.
- Etehadnia, M. (2009). Salt Stress Tolerance in Potato Genotypes. Ph. D Thesis. University of Saskatchewan. Canada. P. No. 244.
- Felger, R., Rutman, S & Malusa, J. (2014). Ajo Peak to Tinajas Altas: Flora of Southwestern Arizona: Part 8, Eudicots: Acanthaceae–Apocynaceae. *Phytoneuron*. 85: 1–71.
- Food and Agriculture Organization the United Nations (FAO). (2010). Farming without Land, Fishing without Water: Gaza Agriculture Sector Struggles to Survive. Palestine.
- Franz, S., Ehlerta, B., Liese, A., Kurth, J., Cazale, A & Romeis, T. (2011). Calcium-Dependent Protein Kinase CPK21 Functions in Abiotic Stress Response in *Arabidopsis thaliana*. *Molecular Plant*. 4: 83- 96.
- Gandonou, C., Errabii, T., Abrini, J., Idaomar, M & Senhaji, N. (2006). Selection of Callus Cultures of Sugarcane (*Saccharum* sp.) Tolerant to NaCl and their Response to Salt Stress. *Plant Cell Tiss Organ Cult*. 87: 9- 16.
- George, E., Hall, M & Klerk, G. (2008). Plant Propagation by Tissue Culture, Third Edition, Springer, Netherlands. P. No. 3, 13, 34, 43, 44, 175, 335
- Gol, D. (2006). Physiology and Genetic Characterization of Salt Tolerance in tomato ((*Lycopersicon esulentum*). Master Thesis. Izmir University. Turkey. P. No. 54.
- Golldack, D & Dietz, K. (2001). Salt-Induced Expression of the Vacuolar H1-ATPase in the Common Ice Plant Is Developmentally Controlled and Tissue Specific1. *Plant Physiology*. 125: 1643–1654.
- Guse, W & Larsen, F. (2001). Propagating Herbaceous Plants from Cuttings. A Pacific Northwest Extension Publication, USA.

- Hall, R. (1999). Plant Cell Culture Protocols (Methods in Molecular Biology). First Edition. Humana Press. United State of America. P. No. 7, 12.
- Hamdan, S. (2012). Artificial Recharge of Groundwater with Stormwater as a New Water Resource- Case Study of the Gaza Strip, Palestine. Ph. D Thesis. Berlin University. German. P. No. 7.
- Hanks, S & Hunter, T. (1995). The Eukaryotic Protein Kinase Superfamily: Kinase (catalytic) Doman Structure and Classification. *FASEB Journal*. 9: 576- 596.
- Haouala, F. & Jaziri, F. (2009). *In Vitro* Propagation of Carnation (*Dianthus caryophyllus* L.) Under Salt Stress. *Pak. J. Biotechnol*. 6: 27-30.
- Hong, S., Jon, J., Kwak, J & Nam, H. (1997). Identification of a receptor-like Protein Kinase Gene Rapidly Induced by Abscisic acid, Dehydration, High salt, and cold Treatments in *Arabidopsis thaliana*. *Plant Physiol*. 4: 1203–1212.
- https://plants.usda.gov/java/ClassificationServlet?source=display&classid=DICA accessed in 26-28/10/2014- 10:10pm.
- Ichimura, K., Mizoguchi, T., Yoshida, R., Yuasa, T & Shinozaki, K. (2000). Various Abiotic Stresses rapidly Activate Arabidopsis MAP kinases ATMPK4 and ATMPK6. *The Plant Journal*. 24: 655- 665
- Idowu, A., Ibitoye, O & Ademoyegun, O. (2009). Tissue Culture as A plant Production Technique for Horticultural Crops. *African Journal of Biotechnology*. 8: 3782- 3788.
- Ilahi, I., Aziz, F & Jabeen, M. (1995). Tissue Culture Studies for Micropropagation of Carnation (*Dianthus caryophyllus* L.). *Pak. J. Bot.* 27: 411- 415.
- Ishitani, M., Xiong, L., Stevenson, B & Zhul, J. (1997). Genetic Analysis of Osmotic and Cold Stress Signal Transduction in Arabidopsis: lnteractions and Convergence of Abscisic Acid-Dependent and Abscisic Acid-lndependent Pathways. *The Plant Cell*. 9:1935-1949.
- Jaggi, D. (2013) A micropropagation System for Carnation (*Dianthus caryophyllus*) an Important Ornamental plant. Master Thesis. Thapar University. Patiala. P. No. 19, 25, 27.

- Jampeetong, A & Brix, H. (2009). Effects of NaCl Salinity on Growth, Morphology, Photosynthesis and Proline Accumulation of *Salvinia natans*. *Aquatic Botany*. 91: 181- 186.
- Jawaharlal, M., Ganga, M., Padmadevi, R., Jegadeeswar, V. and Karthikeyan, S. (2009). A technical Guide on Carnation. First Edition. Tamil Nadu Agricultural University. India. P. No. 1.
- Jiménez, B. (2006). Irrigation in Developing Countries Using Wastewater. *International Review for Environmental Strategies*. 6: 229- 250.
- Jogaitė, V., Lazutka, R., Stapulionytė, A., Naujalis, J., Odland, A & Bjerketvedt, D. (2006). Analysis of DNA Polymorphism in Wild Populations of Herb-Paris (*Paris quadrifolia L*., Trilliaceae) from Lithuania and Norway. *BIOLOGIJA*. 1: 18- 23.
- Khachatourians, G., Hui, Y., Scorza, R & Nip, W. (2002). Transgenic Plants and Crops. First Edition. Marcel Dekler, Inc. New York. P. No. 88, 89, 90.
- Khaleda, L., Ahmed, A., Marzan, L & Forkan, M. (2007). Identification of Callus Induction and Plant Regeneration Responsiveness in Presence of NaCl in *In Vitro* Culture of Some Deepwater Rice (*Oryza sativa* L.) Cultivars. *Asian Journal of Plant Sciences*. 6: 36- 41.
- Khan, M., Ahmad, D., Adnan, M & Khan, M. (2014). The Effect of Somaclonal Variation on Salt Tolerance and Glycoalkaloid Content of Potato Tubers. *AJCS*. 8: 1597- 1606.
- Khan, N., Khan, M., Asgher, M., Fatma, M., Masood, A & Syeed, S. (2014). Salinity Tolerance in Plants: Revisiting the Role of Sulfur Metabolites. *Plant Biochem Physiol*. 2: 1- 8.
- Khavarinejad, R & Mostofi, Y. (1998). Effect of NaCl on Photosynthetic Pigments, Saccharides, and Chlorophyll Ultrastructure in Leaves of Tomato Cultivars. *Photosynthetica*. 35: 151- 154.
- Khorami, R & Safarnejad, A. (2011). *In Vitro* Selection of *Foeniculum vulgare* for Salt Tolerance. *Notulae Scientia Biologicae*. 3:90- 97.
- Kong, X., Lv, W., Jiang, S., Zhang, D., Cai, G., Pan, J & Li, D. (2013). Genome-Wide Identification and Expression Analysis of Calcium-Dependent Protein Kinase in Maize. *BMC Genomics*. 14: 1- 15.

- Larudee, M. (2011). Gaza – Palestine: Out of the Margins. First Edition. Ibrahim Abu-Lughod Institute of International Studies– Birzeit University. Palestine. P. No. 142.
- Leva, A & Rinaldi, L. (2012). Recent Advances in Plant *In Vitro* Culture. First Edition. InTech. Croatia. P. No. 8, 9, 32, 34, 63, 65, 94.
- Libik, M., Pater, B., Elliot, S & Slesaka, I. (2004). Malate Accumulation in Different Organs of *Mesembryanthemum crystallinum* L. Following Age-dependent or Salinity-triggered CAM Metabolism. *Z. Naturforsch*. 59: 223-228.
- Longstreth, D & Nobel, P. (1979). Salinity Effects on Leaf Anatomy. *Plant Physiol*. 63: 700-703.
- Maathuis, F. (2013). Sodium in plants: Perception, Signalling, and Regulation of Sodium Fluxes. *Journal of Experimental Botany*. 10: 1- 10.
- Madi, M. (2001). Wild Plants of the Costal Sand Dunes of Gaza strip. First Edition. Mekdad. Palestine. P. No. 1.
- Mahajan, S & Tuteja, N. (2005). Cold, Salinity and Drought Stresses: An Overview. *Archives of Biochemistry and Biophysics*. 444: 139- 158.
- Mansour, M., Salama, K & Al-Mutawa, M. (2003). Transport Proteins and Salt Tolerance in Plants. *Plant Science*. 164: 891- 900.
- Messerschmid, C. (2011). Water in Gaza: Problems and Prospects.
- Mian, A., Senadheera, P & Maatuis, F. (2011). Improving Crop Salt Tolerance: Anion and cation tramsporter Engineering Tatgets. *Plant Press*. 5: 64- 72.
- Mihaljević, I., Dugalić, K., Tomaš, V., Viljevac, M., Pranjić, A., Čmelik, Z., Puškar, B & Jurković, Z. (2013). *In vitro* Sterilization Procedures for Micropropagation of Oblacinska Sour cherry. *Journal of Agricultural Sciences.* 58: 117- 126.
- Morris, D & Duretto, M. (2009). Flora of Tasmania. *Tasmania Museum and Art Gallery*. 1: 1- 10.
- Munns, R., Husain, S., Rivelli, A., James, R & Condon, A. (2002). Avenues for Increasing Salt Tolerance of Crops, and the Role of Physiologically Based Selection Traits. *Plant and Soil.* 247: 93–105.

- Nabors, M. (1990). Environmental Stress Resistance. In: Plant cell Lines Selection, Procedures and Applications. First Edition. P.J. Weinheim, Basel Cambridge. New York. P. No. 168, 185.
- Nakayama, H., Horie, T., Yonamine, I., Shinmyo, A & Yoshida, K. (2005). Improving salt tolerance in plant cells. *Plant Biotechnology*. 22: 477- 487.
- Nelson, D., Rammesmayer, G & Bohnert, H. (1998). Regulation of Cell-Specific Inositol Metabolism and Transport in Plant Salinity Tolerance. *The Plant Cell*. 10: 753–764.
- Osakabe, Y., Maruyama, K., Seki, M., Satou, M., Shinozaki, K & Shinozaki, K. (2005). Leucine-Rich Repeat Receptor-Like Kinase1 Is a Key Membrane-Bound Regulator of Abscisic Acid Early Signaling in Arabidopsis. *The Plant Cell*. 17:1105- 1119
- Osakabe, Y., Mizuno, S., Tanaka, H., Maruyama, K., Osakabe, K., Todaka, D., Fujita, Y., Kobayashi, M., Shinozaki, K & Shinozaki, K. (2010). Overproduction of the Membrane-bound Receptor-like Protein Kinase 1, RPK1, Enhances Abiotic Stress Tolerance in *Arabidopsis*. *Journal of Biological Chemistry*. 285: 9190- 9201.
- Osakabe, Y., Shinozaki, K., Shinozaki, K & Tran, L. (2013). Sensing the Environment: key Roles of Membrane- Localized Kinases in Plant Perception and Response to Abiotic Stress. *Journal of Experimental Botany*. 64: 445–458.
- Palestine Trade Center (PTC). (2009). Gaza Crossings Monthly Monitoring Report. Palestine- http://www.lacs.ps/documentsShow.aspx?ATT_ID=2011.
- Palestinian Water Authority (PWA). (2012). Annual Status Report on Water Resources, Water Supply and Wastewater in the Occupied State of Palestine 2011. Palestine.
- Pandey, M & Chikara, S. (2014). *In vitro* Regeneration and Effect of Abiotic Stress on Physiology and Biochemical Content of *Stevia Rebaudiana* 'Bertoni'. *J Plant Sci Res*. 1: 1- 9.
- Paranychianakis, N & Chartzoulakis, K. (2005). Irrigation of Mediterranean Crops with Saline Water: from Physiology to Management Practices. *Agriculture, Ecosystems & Environment*. 106: 171-187.
- Pareek, A., Kantia, A & Kothari, S. (2004) *In vitro* cloning of Ornamental Species of *Dianthus*. *Indian Journal of Biotechnology*. 3: 263- 266.

- Parida, A & Das, A. (2005). Salt Tolerance and Salinity Effects on Plants: a review. *Ecotoxicology and Environmental Safety*. 60: 324–349.
- Patel, M. (2003). Medical sterilization methods. LEMO USA, inc.
- Pessarakli, M. (2001). Handbook of Plant and Crop Physiology. Second Edition. Marcel Dekker, Inc, NewYork. P. No. 139, 569.
- Quelros, F., Fidalgo, F., Santos, I & Salema, R. (2007). *In vitro* Selection of Salt Tolerant Cell Lines in *Solanum tuberosum* L. *Biologia Plantarum* 51: 728-734.
- Rai, A & Takabe, T. (2006). Abiotic Stress Tolerance in Plants: Toward the Improvement of Global Environment and Food. First edition. Springer, Netherlands. P. No. 13.
- Rai, M., Kalia, R., Singh, R., Gangola, M & Dhawan, A. (2011). Developing Stress Tolerant Plants Through *In Vitro* Selection- An Overview of the Recent Progress. *Environmental and Experimental Botany*. 71: 89- 98.
- Rao, K., Raghavendra, A & Reddy, K. (2006). Physiology and Molecular Biology of Stress Tolerance in Plants, First Edition, Springer, Netherlands. P. No. 43, 68, 75.
- Rao, S & FTZ, J. (2013). *In vitro* Selection and Characterization of Polyethylene Glycol (PEG) Tolerant Callus Lines and Regeneration of Plantlets from the Selected Callus Lines in Sugarcane (*Saccharum officinarum* L.). *Physiol Mol Biol Plants*. 19: 261–268.
- Rao, S & Patil, P. (2012). *In Vitro* Selection of Salt Tolerant Calli Lines and Regeneration of Salt Tolerant Plantlets in Mung Bean (*Vigna radiata* L. Wilczek). Biotechnology - Molecular Studies and Novel Applications for Improved Quality of Human Life. *InTech*. 197- 213.
- Riaz, A., Younis, A., Taj, A., Karim, A., Tariq, U., Munir, S & Riaz, S. (2013). Effect of Drought Stress on Growth and Flowering of Marigold (*Tagetes edecta* L.). *Pak. J. Bot*. 45: 123-131.
- Rodr´ıguez, P., Torrecillas, A., Morales, M., Ortuño, M & Sánchez-Blanco**,** M. (2005). Effects of NaCl Salinity and Water Stress on Growth and Leaf Water Relations of *Asteriscus maritimus* plants. *Environmental and Experimental Botany*. 53: 113-123.

- Rodríguez, M., Canales, E & Borrás-Hidalgo, O. (2005). Molecular Aspects of Abiotic Stress in Plants. *Biotecnología Aplicada.* 22: 1-10.
- Roodbaraky, F., Hashemabad, D & Vand, S. (2012). Effect of Salicylic Acid on Vase Life of Cut Carnation (*Dianthus caryophyllus* L. cv. 'Liberty Abgr'). *Annals of Biological Research.* 3: 5127- 5129.
- Rout G, Mohapatra, A & Jain, S. (2006). Tissue Culture of Ornamental Pot Plant: A critical Review on Present Scenario and Future prospects. *Biotechnology Advances.* 24: 531–560.
- Russell, B, Rathinasabapathi, B & Hanson, A. (1998). Osmotic Stress Induces Expression of Choline Monooxygenase in Sugar Beet and Amaranth. *Plant Physiol*. 116: 859- 865.
- Rutala, M., Weber, D and the Healthcare Infection Control Practices Advisory Committee (HICPAC). (2008). Guideline for Disinfection and Sterilization in Healthcare Facilities. Department of health and human services. USA.
- Ryan, J. (2008). Understanding and managing cell culture contamination. Technical Bulletin. Life science. USA. 1- 24.
- Saijo, Y., Kinoshita, N., Ishiyama, K., Hata, S., Kyozuka, J., Hayakawa, T., Nakamura, T., Shimamoto, K., Yamaya, T & Izui, K. A Ca^{2+} - Dependent Protein Kinase that Endows Rice Plants with Cold- and Salt- Stress Tolerance Functions in Vascular Bundles. (2001). *Plant Cell Physiol*. 42: 1228- 1233.
- Sairam, R & Tyagi A. (2004). Physiology and Molecular Biology of Salinity Stress Tolerance in Plants. *Current Science*. 86: 407- 421.
- Sajid, Z & Aftab, F. (2014). Plant Regeneration from *In Vitro*- Selected Salt Tolerant Callus Cultures of *Solanum tuberosum* L. *Pak. J. Bot*., 46: 1507- 1514.
- Scocchi, A., Faloci, M., Medina, R., Olmos, S & Mroginski, L. (2004). Plant Recovery of Cryopreserved Apical Meristem-tips of *Melia azedarach* L. using Encapsulation/ Dehydration and Assessment of their Genetic Stability. *Euphytica* 135: 29- 38.
- Shafi, M., Guoping, Z., Bakat, J., Khan, M., Islam, E., Khan, M & Raziuddin. (2010). Effect of Cadmium and Salinity Stress on Root Morphology of Wheat. *Pak. J. Bot*. 42: 2747-2754.

- Shahid, M., Pervez, M., Balal, R., Ahmad, R., Ayyub, C., Abbas, T & Aktar, N. (2011). Salt Stress Effects on Some Morphological and Physiological Characteristics of Okra (*Abelmoschus esculentus* L.). *Soil Environ.* 30: 66-73, 2011.
- Shahpiri, A. (2008). Hormone-dependence of Gene and Protein Expression in Barley Aleurone Layer and Characterization of NADPH-dependent Thioredoxin Reductase/Thioredoxin System in Barley Seeds. Ph. D Thesis Technical University of Denmark. Denmark. P. No. 21, 23.
- Sharma, A & Agrawal, V. (2012). Tissue Culture Aspects of Ornamental Plants. *CIBTech Journal of Biotechnology*. 1: 40- 48.
- Shenker, M., Harush, D., Ben- Ari, J & Chefetz, B. (2011). Uptake of Carbamazepine by Cucumber plants – A case Study Related to Irrigation with Reclaimed Wastewater. *Chemosphere*. 82: 905–910.
- Shi, H., Ishitani, M., Kim, C & Zhu, J. (2000). The *Arabidopsis thaliana* Salt Tolerance Gene SOS1 Encodes A putative Na^+/H^+ Antiporter. *PNAS*. 97: 6896–6901.
- Shiu, S & Bleecker, A. (2001). Plant Receptor-Like Kinase Gene Family: Diversity, Function, and Signaling. *Science's STKE*. 113: 1- 13.
- Singh, G. (2010). Plant Systematics An integrated Approach. Third Edition. Science Publishers, Enfield, NH, USA. P. No. 536.
- Smith, R. (2013). Plant Tissue Culture: Techniques and Experiments. Third Edition. Elsevier Inc. United State of America. P. No. 26.
- Srivastava, A.K., Bhargava, P., Kumar, A., Rai, L.H & Neilan, L.A. (2009). Molecular Characterization and the Effect of Salinity on Cyanobacterial Diversity in the Rice Fields of Eastern Uttar Pradesh, India. *Saline system.* 5: 1- 17.
- Stewart, C. (2008). Plant Biotchnology and Genetics: Principle, Techniques and Aplication. John Wiley & Sons, Inc. Canada. P. No. 115, 116, 126 127, 128.
- Su, S & An, L. (2010). Isolation of Coline Monooxygenase (CMO) Gene from *Salicorina europaea* and Enhanced Salt Tolerance of Transgenic Tobacco with CMO genes. *Indian Journal of Biotechnology & Biophysics.* 47: 298- 305.

- Tah, J & Mamgain, A. (2013). Review- Variation in Different Agronomical Characters of Some Carnation (*Dianthus caryophyllus*) Cultivars. *Research Journal of Biology*. 1: 10- 23.
- Taiz, L & Zeiger, E. (2006). Plant Physiology. Fourth Edition. Sinauer, Sunderland, Mass, USA. P. 33, 612.
- Takahashi, R., Liu, S & Takano, T. (2009). Isolation and Characterization of Plasmamembrane Na^+/H^+ antiporter Genes from Salt-Sensitive and Salt-Tolerant Reed Plants. *Journal of Plant Physiology*. 166: 301- 309.
- Tam, D & Lang, N. (2003). *In vitro* Selection for Salt Tolerance in Rice. *Omonrice* 11: 68-73.
- Tarannum, MS & Naik, B (2014). Performance of Carnation (*Dianthus caryophyllus* L.) Genotypes for Qualitative and Quantitative Parameters for Assess Genetic Variability among Genotypes. *American International Journal of Research in Formal, Applied & Natural Sciences*. 5: 96-101.
- Tena, G., Asai, T., Chiu, W & Sheen, J. (2001). Plant Mitogen- ctivated Protein Kinase Signaling Cascades. *Current Opinion in Plant Biology*. 4: 392–400.
- Thomas, T. (2008). The Role of Activated Charcoal in Plant Tissue Culture. *Biotechnology Advances*. 26: 618- 631.
- Tirajoh, A. (2005). Isolation and Characterization of Novel Salt- Responsive Genes from Tomato (*Lycopersicon esulentum* Mill) root. Ph.D Thesis. Simon Fraser University. Canada. P. No. 304.
- Ubeid, K. (2011). The Nature of the Pleistocene Holocene palaeosols in the Gaza Strip, Palestine. *Geologos*. 17: 163- 173.
- United Nations Environment Programme (UNEP). (2009). Environmental Assessment of the Gaza Strip. First Edition. United Nations Environment Programme. Palestine. P. No. 56.
- Vargas, V & Flota, F. (2006). Plant Cell Culture Protocols. Second Edition. Humana Press Inc., United State of America. P. No. 4, 9.
- Vasil, I & Thrope, T. (1994). Plant Cell and Tissue Culture. First Edition. Springer. Netherlands. P. No. 46.
- Walker, J & Ralpley, R. (2008). Molecular Biomethods Handbook (Methods in Molecular Biology). Second Edition. Humana Press. USA. P. No. 875, 877.

- Wang, H., Hsieh, Y., Harwell, M & Huang, W. (2007). Modeling Soil Salinity distribution along topographic gradients in tidal salt marshes in Atlantic and Gulf coastal regions. *Ecological modeling*. 201: 429- 439.
- Wang, K. (2006). *Agrobacterium* Protocols. Second Edition. Volume 2. Humana Press Inc. United States of America. P. No. 313.
- Wang, S., Zhang, Y., Perez, P., Deng, Y., Li, Z & Huang, D. (2011). Isolation and Characterization of a vacuolar Na^+/H^+ Antiporter Gene from *Cucumis melo* L. *African Journal of Biotechnology*. 10: 1752-1759.
- Wang, W., Vinocur, B & Altman, A. (2003). Plant Responses to Drought, Salinity and Extreme Temperatures: Towards Genetic Engineering for Stress Tolerance. *Planta*. 218: 1- 14.
- Wang, Z., Li, P., Fredricksen, M., Gong, Z., Kim, C., Zhang, C., Bohnert, H., Zhu, J., Bressan, R., Hasegawa, P., Zhao, Y., Zhang, H. (2004). Expressed Sequence Tags from *Thellungiella halophila*, a new Model to Study Plant Salt-Tolerance. *Plant Science*. 166: 609–616.
- Wilson, C & Shannon, M. (1995). Salt-Induced Na+/H+ Antiport in Root Plasma Membrane of A glycophytic and Halophytic species of Tomato. *Plant Science*. 107: 147- 157.
- Wright, K & Northcote, D. (1973). Differences in Ploidy and Degree of Intercellular Contact in Differentiating and Non Differentiating Sycamore calluses. *J. Cell Sci.* 12: 37- 53.
- Xiong, L & Yang, Y. Disease Resistance and Abiotic Stress Tolerance in Rice Are Inversely Modulated by an Abscisic Acid–Inducible Mitogen-Activated Protein Kinase. *The Plant Cell*. 15: 745- 759.
- Xu, J., Wu, L., Chang, A & Zhang, Y. (2010). Impact of Long-Term Reclaimed Wastewater Irrigation on Agricultural Soils: A preliminary Assessment. *Journal of Hazardous Materials*. 183: 780- 786.
- Yaacob, J., Taha, R & Esmaeili, A. (2013). Comparative Studies on Cellular Behaviour of Carnation (*Dianthus caryophyllus* Linn. cv. Grenadin) Grown *In Vivo* and *In Vitro* for Early Detection of Somaclonal Variation. *Hindawi Publishing Corporation*. 8: 1- 9.

- Yang, J & Yen H. (2002). Early Salt Stress Effects on the Changes in Chemical composition in Leaves of Ice Plant and Arabidopsis. A Fourier transform infrared spectroscopy study. *Plant Physiology*. 130: 1032- 1042.
- Yokoi, S., Bressan, R & Hasegawa, P. (2002). Salt Stress Tolerance of Plants. *JIRCAS Working Report.* 1: 25-33.
- Youssef, A. (2009). Salt Tolerance Mechanisms in Some Halophytes from Saudi Arabia and Egypt. *Research Journal of Agriculture and Biological Sciences*. 5: 191- 206.
- Yu, J., Huang, J., Wang, Z., Zhang, J & Chen, S. (2007). An $Na^{+/}H^{+}$ Antiporter Gene from Wheat Plays an Important Role in Stress Tolerance. *J. Biosci.* 32: 1153- 1161.
- Zagorchev, L., Seal, C., Kranner, I & Odjakova, M. (2013). A Central Role for Thiols in Plant Tolerance to Abiotic Stress International. *Journal of Molecular Sciences.* 14: 7405- 7432.
- Zahid, M., Hasan, M., Adil, M., Hossain, M & Mian M. (2014). *In Vitro* Screening for Salt Tolerance in Aromatic Rice Genotypes. *Open Science Journal of Bioscience and Bioengineering*. 1: 28- 32.
- Zan, Y., Ji , Y., Zhang, Y., Yang, S., Song, Y & Wang, J. (2013). Genome-Wide Identification, Characterization and Expression Analysis of Populus Leucine-Rich Repeat Receptor-Like Protein Kinase Genes. *BMC Genomics*. 14: 1- 14.
- Zhang, C., Zhao, Ge, W., Zhang. Y., Song. Y., Sun, D & Gu, Y. (2011). An Apoplastic H-Type Thioredoxin Is Involved in the Stress Response through Regulation of the Apoplastic Reactive Oxygen Species in Ric *Plant Physiol*. 157: 1884- 1899.
- Zhu, J. (2001). Plant salt tolerance. *TRENDS in Plant Science*. 6: 66- 71.
- Zhu, J. (2003). Regulation of Ion Homeostasis Under Salt Stress. *Current Opinion in Plant Biology*. 6: 441- 445.
- Zinnah, K., Zobayer, N., Sikdar, S., Liza, L., Chowdhury, M and Ashrafuzzaman, M. (2013). *In Vitro* Regeneration and Screening for Salt Tolerance in Rice (*Oryza sativa* L.). *Research Journal of Biological Sciences*. 2: 29- 36.

- Zou, J., Wei, F., Wang, C., Wu, J., Ratnasekera, D., Liu & Wu, W. (2010). Arabidopsis Calcium-Dependent Protein Kinase CPK10Functions in Abscisic Acid- and Ca^{2+} -Mediated Stomatal Regulation in Response to Drought Stress. *American Society of Plant Biologists*. 154: 1232- 1243.

APPENDIX

Appendix I

(100 mL)50X TAE (Tris acetate ethyldimethyl tetra acetic acid) Buffer

Tris base	24.2 g
Glacial acetic acid	5.71 mL
0.5M EDTA (pH 8.0)	10 mL

The pH of diluted solution (1X) was ~8.5

Appendix II

Node Explants Stock Solutions, Antibiotics and Culture Media

Stock Solutions

- Thidiazuron (TDZ) stock (100 mg/L): dissolve 10 mg of TDZ in a few drops of 1 *N* NaOH and make up the volume to 100 mL with double distilled water (ddH2O) and store at −20°C.
- α-napthalene acetic acid (NAA) stock (100 mg/L): Dissolve 10 mg of NAA in a few drops of 1 *N* NaOH and make up the volume to 100 mL with ddH_2O and store at −20°C.
- *N*6-benzyladenine (BA) stock (100 mg/L): Dissolve 10 mg of BA in a few drops of 1 *N* NaOH and make up the volume to 100 mL with ddH2O and store at −20°C.

Antibiotics

All antibiotics was dissolved either in ddH_2O, filter-sterilized, and stored in aliquots in −20°C. They should be added to media after autoclaved and cooled down to around 55°C.

- Kanamycin stock (50 mg/mL).
- Hygromycin (50 mg/mL).

Culture Media

Medium I: Murashige and Skoog (MS) Himedia (Cat. No. PT018) medium.

Component	Volume (mg/L)
NH_4NO_3	1650
KNO_3	1900
$MgSO_4 \cdot 7H_2O$	180.69
KH_2PO_4	170

H_3BO_3	6.2
$MnSO_4 \cdot 4H_2O$	16.90
$ZnSO_4 \cdot 7H_2O$	8.6
$CoCl_2 \cdot 6H_2O$	0.025
$CuSO_4 \cdot 5H_2O$	0.025
KI	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.25
$FeSO_4 \cdot 7H_2O$	27.8
$Na_2EDTA \cdot 2H_2O$	37.3
myo-inositol	100
thiamine HCl	0.1
pyridoxine HCl	0.5
nicotinic acid	0.5
glycine	2.0
TDZ	1
sucrose	20,000
Phytagel	2000

Chemicals were added to the medium 440 mg/L $CaCl_2 \cdot 2H_2O$, 189.31 mg/L $MgSO_4 \cdot 7H_2O$ and 5.4 mg/L $MnSO_4 \cdot 4H_2O$. The addition were based on based previously published, (Wang, 2006).

The pH was adjusted to 5.75 using NaOH, with and without charcoal and the medium was autoclaved at 121°C for 15 min.

Hint: Sodium chloride was added to medium I in the concentration of 0, 25, 50, 100, 150 and 200 Mm

Medium II:

Medium II, a: Murashige and Skoog (MS) medium as in medium I was supplemented with 4 mg/L BA , and 30 g/L sucrose. pH was adjusted to 5.71 ± 0.5 using NaOH, added 2 g/L Phytagel, and autoclaved at 121°C for 15 min, (Wang, 2006; Ali et al., 2010).

Medium II, b: MS medium as in medium I and II except the conc. of hormones added was 0.5 mg/L NAA + 1.0 mg/L BA and the pH was adjusted to 5.8, (Pareek et al., 2004; Wang, 2006).

Hint: Sodium chloride added to medium II in the concentration of 0, 50 and 100 mM.

Medium III:

Murashige and Skoog (MS) medium as in medium I was supplemented with 1 mg/L BA , and 30 g/L sucrose. pH was adjusted to 5.71 ± 0.5 using NaOH added 2 g/L Phytagel, and autoclaved at 121°C for 15 min, (Wang, 2006; Ali et al., 2010).

Hint: Sodium chloride added to medium III in the concentration of 0, 50 and 100 mM.

Medium IV

Murashige and Skoog (MS) medium as in medium I was supplemented with 1 mg/L NAA , and 30 g/L sucrose. pH was adjusted to 5.71 ± 0.5 using NaOH added 2 g/L Phytagel, and autoclaved at 121°C for 15 min, (Wang, 2006; Ali et al., 2010).

Hint: Sodium chloride added to medium II in the concentration of 0, 50 and 100 mM.

Appendix III

The Electrical Conductivity (EC) of the NaCl conc.

Sodium chloride conc. (mM)	**EC (dS//m)**
50	**5**
100	**10**
150	**15**
200	**20**

For every 10 Mm, the EC is equal 1 dS/m

APPENDIX IV

Limitation of the research

The researcher faced number of problems before and after working in this research, which summarized as the following;

- Before working in this research the researcher decided to make genetic modification for *Dianthus caryophyllus* L. (omagio) and the researcher succeeded to take the osmotin gene from Professor K. C .Bansal Professor (Molecular Biology and Biotechnology), Indian Agricultural Research Institute, but because of unknown reasons the transferring was not completed.
- The researcher decided to isolate the osmotin gene or any gene activated under abiotic stress (salt stress) and later use it in the genetic transformation. In order to apply her decision, the researcher studied and surveyed the halophyte plants which presented in Gaza strip. This took a lot of months so the researcher could be able to study, survey and choose the halophyte plant which later detected as (*Mesembryanthemum crystallinum* L.).
- The decision to choose *M. crystallinum* L. in this study was based on the earlier study in this field. The choice was successful because this plant have the ability to resist high concentration of salt and during stress many genes could be activated.
- With all problems the researcher insist to choose this research. Later she made the orders to different companies to buy the materials and kits for the work but due to the insecure situation in Gaza strip, researcher had to wait more than 6 to 8 months. The materials, kits which ordered was not arrived in same times. This also, delayed some experiments. For example, if any one need to make experiment (A for example) and the materials for his or her work (in two orders, two companies) are not arrived, what shall he or she make?. He or she will try to get the materials from some laboratory in Islamic University or other places. If this trials did not completely succeed (because the genetic modification technique dose not exist in Gaza strip), the researcher would wait a lot of times in order to receive his or her materials and kits.

- Later and based on the earlier studies, the researcher decided to isolate Na^+/H^+ antiporter gene (*nhx1*), but due to genetic variation among of plant species *M. crystallinum* L., other genes were isolated (putative thioredoxin) and (serine/threonine-protein kinase EDR1). Because the isolated genes are not as the study aimed previously, the researcher became have not appropriate materials to be able to complete the transformation steps. Thus, in order to make the genetic transformation of carnation and complete the first decision (to get modified plant have the ability to cope salt stress), the researcher need new materials

Also, The researcher faced number of troubleshooting during this study, which delayed the steps of this work (study plan). The problems summarized as the following:

- **Troubleshooting in the autoclave instrument;**
  The researcher overcame this problem using the sterilization of the materials and media in other departments of Islamic University of Gaza before the biology and biotechnology department brought another instrument.

- **Troubleshooting in the Nano photometer instrument;**
  The uses of this device in the research focuses on the detection of the concentration of RNA and DNA samples and to quantify the purity of these samples. The trouble is this device broke down which delayed the sequencing step. The problem was overcome by detection of the concentration in Al- Aqsa University.
  Note: this problem appeared in the DNA production step. The detection of DNA conc. made a timing gap between the PCR production step in Islamic University of Gaza and the detection of conc. in Al- Aqsa University (Al-Aqsa University brought this device after one year).

- The sequencing of the PCR products was analyzed after more than 6 months of detection of the conc. of the PCR products in Al- Aqsa University due to;
  1. Shipping companies in Gaza rejected the transferring of biological samples from Gaza to any place outside Gaza strip.

2. Insecure situation of Gaza strip, which delaying persons outside traveling.

- **Troubleshooting in plant growth chamber;**

This device is used to cultivate and propagate the plant cells and tissues under controlled conditions. The device started working after 8 months from the researcher start the work in the research. The problems which appeared in this study (which took months) are;

1. the bad power situation in Gaza strip, so, the programming of this device was continuously lost.

   Note: the programming of this instrument is divided into two cycles(16 h day and 8 h night). If the power became off, the programming will be lost. This made the instrument with just one cycle either 24 h night or 24 h day.
2. the temperature of the device was continuously elevating, the cells and tissues was being destroyed. This made the researcher repeated some steps and sometimes start from first step. Also, the *Dianthus caryophyllus* L. (omagio) and the MS media (with repeated steps) were fully consumed. So the research waited new cultivar in another season wasting more time.
3. not completely solving the irregular cooling and heating (troubleshooting in the motor of the device) by the specialist engineers and physicians, the new cultivar which the researcher succeeded to get under *in vitro* and *ex vitro* conditions died (in last step before transferring it to the greenhouse).

The researcher decided previously to detect the genetic variation between the callus, plantlet and full plant via inter simple sequence repeat markers (ISSR-PCR). This techniques are used to detect the genetic stability of new cultivar of carnation. Also, the researcher decided previously to do some physiological test like (H_2O_2 and prolin determination) and to test the accepting of new cultivar of the reclaimed waste water in order to minimize the using of ground water. All of these decisions was not applied due to continuance

troubleshooting of plant growth chamber which made the researcher busy to refresh her cells after every troubleshooting.

APPENDIX V

Middle East Desalination Research Center
Interim Budget Report (in US$)

Principle Investigator:	Nisreen Badr	Project No:	13-CoE-003
Project Title:	Enhancement of Salinity Tolerance in *Dianthus caryophyllus* L. (omaggio) using *In vitro* Selection Technique and Isolation of Potential Salinity Tolerance Genes from *Mesembryanthemum crystallinum* L.		
Report Period From:	March, 2014 To: Dec., 2014	Report No.:	2

MATERIALS/ EQUIPMENT (hardware, chemicals, raw materials etc.)

Name of the Item &Purchase Date	Quantity	Unit Price	Reporting Period			Total Previous Periods			Total Expenditure to Date			MEDRC Budget
			Breakdown		Total	Breakdown		Total	Breakdown		Total	
			MEDRC	In-Kind		MEDRC	In-Kind		MEDRC	In-Kind		
Chemical which used from March 2014 to the final research												
wizard sv gel and pcr clean-up kit	1	252.43	252.43								252.43	
100bp DNA Ladder (250µL)	1*2	230.54	461.08								461.08	
Primers	4	18.2425	72.97								72.97	
QIAGEN OneStep RT-PCR Kit	1	722.97	722.97								722.97	
DNA master mix	1	81	81								81	
Phytagel	1	138.378	138.378								138.378	
TDZ	1	635.135	635.135								635.135	
Hygromycin	1	340	340								340	
Kanamycin	1	393	393								393	
α-Naphthaleneacetic acid	1	8	8								8	

6-benzylaminopurine	1	33	33								33	
Sodium hypochlorite	1	29	29								29	
Sucrose	1	16	16								16	
Sodium chloride	1	16	16								16	
Murashige and skoog medium	1	39.29	39.29								39.29	
Phyta jar	1*2	100	200								200	
Sterile bottle	1	132	132								132	
Petri dishes	1	215	215								215	
Sterile loop	200	0.081	16.2								16.2	
Sterile tips (10-200 µL)	1	40.5	40.5								40.5	
1 ml filter tips, sterile	1*2	260	520								520	
Serological pipette 25ml	50	0.43	21.5								21.5	
Tips yellow	1	12	12								12	
Tips blue	1	15	15								15	
Tips blue	1	12	12								12	
Freezing box	1	57	57								57	
Rack for multiple uses	1	4	4								4	
PCR centrifuge tube	1	34	34								34	
PCR racks	1	50	50								50	
Parafilm	1	70	70								70	
Liquid nitrogen	5	13.51	67.55								67.55	
Detachable face	1	1	10								10	

shield												
Sequencing test	4	35	140								140	
Shipping												
From Jaddah to San Diageo for analysis	1	95	95								95	
Wire transfer	2	2*25	50								50	
SUB-TOTAL											5000.003	
Principle investigator (name and signature)	Nisreen Badr											
Principle supervisor (name and signature)	Dr. Abboud El- Kichaoui