Biotechnology Master Program

Palestine Polytechnic University
Deanship of Higher Studies and Scientific Research
Master Program of Biotechnology

Capparis spinosa L. "Caper": *In Vitro* Cultures and Bioassay

By

Ghada M. Haroon Abu Khalaf

In Partial Fulfillment of the Requirements for the Degree
Master in Biotechnology

September, 2011

The undersigned hereby certify that they have read and recommend to the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University and the Faculty of Science at Bethlehem University for acceptance a thesis entitled:

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ABSTRACT

Capparis spinosa L. is one of the known medicinal plants of plant biodiversity in Palestine. It is used in traditional medicine to treat rheumatism and infections. For better exploitation and protection from genetic erosion, this study was conducted to investigate the factors affecting *C. spinosa in vitro* propagation, callus and root culture. Since *C. spinosa* has deep seed dormancy, effects of seed storage and pretreatment with GA_3 were investigated. In order to start motherstock plants, two years old stored seeds gave higher (40%) germination *in vitro* than one year old (6.7%), or newly harvested seeds (0.0%). Pretreatment with GA_3 of fresh harvested seeds did not increase germination percentage. Callus induction was conducted from leaf discs on MS and B5 media supplemented with 0.5 mg/L 2,4-D. MS medium showed highest callus fresh weight compared to B5 medium. Callus tissue was maintained by frequent subculture on MS media with 1.0 mg/L 2,4D and 1.5 mg/L BA under dark condition. Rapid multiplication of caper was achieved by culturing nodal segments on MS medium containing (0.0, 0.5, 1.0, and 2.0 mg/L) zeatin. Zeatin at 0.5 mg/L resulted in the highest shoot height and number of leaves and shoots. Also, the effect of BA (0.0, 0.5, 1.0, 1.5, and 2.0 mg/L) added to MS media containing 0.5 mg/L IAA and 1.0 mg/L GA_3 on shoot multiplication was studied. BA at 0.5 mg/L gave the highest number of leaves and shoots. *In vitro* root culture was induced from leaf discs. MS medium containing IAA, IBA and NAA at the concentrations of 0.0, 0.1, 0.5, and 1.0 mg/L were evaluated for their effects on adventitious root induction. Results revealed that the highest rooting (62.5%) was observed on MS medium with 1.0 mg/L NAA. Moreover, it gave the highest number and length of roots per explants. After six weeks, well established roots were cultured in full-strength MS liquid medium containing 1.0 mg/L NAA and under continuous agitation at 80 rpm. The maximum root growth was obtained after six weeks of culture. Callus, root bark, and leaves of *C. spinosa* were extracted by different solvents like ethanol, apple and grape vinegar. Antimicrobial activity of different

extract types and concentrations (4000, 2000, 1000, 500 µg/disk) was studied using disk diffusion assay. Results under the experiments settings revealed that all caper tested extracts at different concentrations did not show any antimicrobial activities against *E. coli*, *S. aureus*, *C. albicans*, and *C. parapsilosis*.

Key words: *Capparis spinosa* L., Callus, Secondary Metabolites, Root Culture.

زراعة نبات الكبار *Capparis spinosa* L. بالأنسجة ودراسة فاعليته الحيوية.

غادة هارون أبوخلف

ملخص:

نبات الكبار أحد النباتات الطبية المعروفة واحد عناصر التنوع الحيوي النباتي في فلسطين, فهو يستخدم في الطب الشعبي لعلاج الأمراض المعدية والروماتيزم. لحسن استغلال وحماية الكبار من الانجراف الوراثي، أجري هذا البحث لدراسة العوامل التي تؤثر على تكثير الكبار بالأنسجة وزراعة الكالوس والجذور. نظرا لأن بذور الكبار تعاني من طور سكون قوي، فقد تم دراسة تأثير تنضيد البذور والمعالجة بمنظمات النمو مثل GA_3على الإنبات. أظهرت النتائج أن البذور المخزنة لمدة سنتين أعطت نسبة إنبات مئوية مرتفعة (٤٠٪) بالمقارنة بالبذور المخزنة لمدة سنة واحدة (٦,٧%) والبذور المحصودة حديثا (٠,٠ ٪). كما أن معالجة البذور المحصودة حديثا ب GA_3 لم يعزز نسبة الإنبات. من اجل حث إنتاج الكالوس، تم زراعة قطع من أوراق الكبار على نوعين من الوسط الغذائي (MS وB5) المضاف لكل منهما ٠,٥ ملغم/لتر 2,4-D. النتيجة أظهرت أن MS أعطت أعلى وزن للكالوس الطازج بالمقارنة إلى B5. وكما إن أنسجة الكالوس تكاثرت بزراعتها المتكررة على الوسط المغذي MS المحتوي على ١,٠ ملغم/لتر 2,4-D و ١,٥ ملغم/لتر BA في الظلام. من اجل دراسة تأثير التركيز المختلف لzeatin على تشكيل وتكاثر السويقات، زرعت عقل الكبار في الوسط المغذي MS مضاف إليه (٠,٠ ، ٠,٥ ، ١,٠ ، و ٢,٠ ملغم/لتر) من zeatin. وقد بينت النتائج أن ٠,٥ ملغم/لتر من zeatin قد أعطى أكبر عدد من الأوراق والفروع وأعلى ارتفاع للسويقات. أيضا درس تأثير التركيز المختلف ل BA (٠,٠ ، ٠,٥ ، ١,٠ ، ١,٥ ، و ٢,٠ ملغم/لتر) المضافة إلى الوسط المغذي MS المحتوي على ٠,٥ ملغم/لتر من IAA و ١,٠ ملغم/لتر من GA_3 على تشكيل وتكاثر السويقات. النتيجة أظهرت بان ٠,٥ ملغم/لتر من BA أعطى أعلى عدد من الأوراق و الفروع. بالنسبة لحث إنبات الجذور من القطع الورقية في المختبر. درست قدرة (auxins) المختلفة IBA , IAA, NAA على التراكيز المختلفة (٠,٠، ٠,١ ، ٠,٥ ، و ١,٠ ملغم/لتر) المضافة إلى الوسط الغذائي MS على حث الجذور. كشفت النتائج أن الوسط المغذي MS المحتوي على ١,٠ ملغم/لتر من NAA أعطى أعلى نسبة مئوية للجذور (٦٢,٥ ٪)، وأيضا أعطى أعلى عدد من الجذور وأعلى طول للجذور. بعد ست أسابيع من تشكل الجذور، تم زراعة الجذور في الوسط المغذي MS السائل المحتوي على ١,٠ ملغم /لتر NAA وتحت اهتزاز مستمر (٨٠ دورة في الدقيقة) . تم الحصول على أعلى قدر من نمو الجذور بعد ستة أسابيع من الزراعة. المواد الثانوية تم استخلاصها من الكالوس و جذور وأوراق نبات الكبار باستخدام عدة مذيبات مثل الايثانول و خل التفاح و خل العنب. وقد درست أيضا الفعالية الطبية المضادة للميكروبات للمستخلصات المختلفة من الكبار على التراكيز المختلفة مثل (٥٠٠، ١٠٠٠، ٢٠٠٠ ، ٤٠٠٠ ميكروغرام لكل قرص) باستخدام مقياس الانتشار حول القرص. كشفت النتائج أن جميع المستخلصات المختبرة لم تظهر أي فعالية ضد أي نوع من الكائنات الدقيقة المختبرة مثل *E. coli, S. aureus C. albicans*, *C. parapsilosis*

الكلمات المفتاحيه : الكبار، الكالوس، مواد ثانوية، زراعة الجذور.

DECLARATION

I declare that the Master Thesis entitled:

***Capparis spinosa* L. "Caper": *In Vitro* Cultures and Bioassay**

is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

Name and signature: Ghada Haroon Abu Khalaf

Date: 18th September 2011

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Dedication

To the memory of my dear grandfather Dr.Hafez Abdel Nabi and my dear uncle Hajj Adel Abu Khalaf.

To My Parents, for their prayers that were always helping me. I love you, thanks for love, patience and precious support.

To my brothers and sisters for their support and love.

To My nephews and nieces (Haytham, Hiba, Haya, Laila, Leen, and small Laila), thanks for being the happiness of my life.

To my friends Dr. Ghayda` Al Herbawi and Diana Al Saheb for their precious support, advises, and love.

To all of my friends.

Ghada

Acknowledgement

First of all, I would like to express my deep and sincere gratitude to my supervisor Dr. Rami Arafeh for his suggestions, guidance, encouragement, valuable advices and support throughout this study.

I also wish to express my warm and sincere thanks to my co-advisor Dr. Jawad Alzeer for his suggestions, guidance, and advices during my study.

My deep gratitude to the head of Biotechnology Research Center (BRC) Dr. Yaqoub Ashhab and to all staff of the center for their assistance and their genuine and friendly approach.

I express my special thanks to lab technicians in BRC Ms. Asma Tamimi, Mr. Zaid AlTarda, and Mr. Hasan AlTarda for their supports and friendships.

My sincere thanks to my friends and colleagues especially Mohammad Qabajah for his helps in antimicrobial test.

I wish mainly to express my thanks to Caritas Baby Hospital specially the Microbiology Laboratory for offering all the possible assistances for contributing to complete this research. My sincere thanks to Dr Mousa Hindiya and all the persons in lab, especially Raid, Randa, and Isaa.

Last but not least, I would like to express my sincere thanks all my work colleagues for their support and helps.

Big thanks and best wishes to all of you.

Ghada Haroon Abu Khalaf

Abbreviations:

°C	Centigrade
µg	Microgram
µL	Microliter
2,4-D	2,4-Dichlorophenoxyacetic acid
2ip	Isopentenyl adenine
Abs	Absorbance
ANOVA	Analysis of variance
approx.	Approximately
ASE	Accelerated Solvent Extraction
AST	Antimicrobial Susceptibility Test
B5	Gamborg's B5
BA	Benzyl adenine
BSAC	British Society for Antimicrobial Chemotherapy
BC	Before Christ
CFU/ml	Colony-Forming Units per Milliliter
cm	Centimeter
CRD	Completely Randomized Design
cv.	Cultivar
diam.	Diameter
DMSO	Dimethylsulfoxide
et al.	And other
etc.	And so on
EUCAST	European Committee for Antimicrobial Susceptibility Testing
g	Gram
g/L	Gram per liter
GA_3	Gibberellic acid
Gy	Gray
H_2SO_4	Sulfuric acid
HCL	Hydrochloric acid
hr	Hour
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid

i.e.	For example
KOH	Potassium hydroxide
L	Liter
LSD	Least Significant Difference
m	Meter
M	Molar
MAE	Microwave Assisted Extraction
Mcc	McCown woody plants media
mg/L	Milligram per litter
MIC	Minimum Inhibitory Concentration
min	Minute
ml	Milliliter
mM	Millimolar
N	Sample size
NAA	A-naphthaleneacetic acid
NCCLS	National Committee for Clinical Laboratory Science
nm	Nanometer
No.	Number
P	Page
PGR	Plant Growth Regulator
PLE	Pressurized Liquid Extraction
PPFD	Photosynthetic Photon Flux Density
Psi	Pound per square inch
rpm	Rounds per minute
SDW	Sterile Distilled Water
SE	Standard mean error
sec	Second
SFE	Supercritical Fluid Extraction
TDZ	Thidiazuron
TSS	Total Soluble Solid
v/v	Volume by volume
WHO	World Heath Organization
wt	Weight
w/v	Weight by volume

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CHAPTER 1

Introduction

Medicinal plants are considered an important source of medication for thousands of years. The Egyptian pharmacopoeia of Ebers Papyrus described the uses of some medicinal plants like opium and castor oil, and the *Materia medica* of Hippocrates documented the medicinal applications of sage and rosemary (Gossell-Williams et al., 2006). Today all over the world, there is a growing interest for using medicinal plants as natural sources in pharmaceutical and food industries. According to the World Heath Organization (WHO) estimations, 80% of the worlds' population mainly in some Asian and African countries and less in developed countries depends on herbal medicine for their primary health care needs (WHO, 2008). Moreover, medicinal plants have been used for their antihypertensive, anti-asthmatic, anti-diabetic, antimicrobial activities for centuries and today their scientific justification was provided by recognition and isolation of bioactive phytochemicals (Kaçar, 2008).

In Palestine, about 2780 plant species are recorded as native or naturalized (Ali-Shtayeh et al., 2008); where more than 700 plant species are used for their medicinal properties and 129 plant species are still used in Arabic traditional medicine to treat many diseases (Jaradat, 2005; Said et al., 2002). Many of wild plants are becoming endangered due to rural and urban extension, uncontrolled deforestation and illegal collection, industrial pollution, and low level of awareness in the Palestinian population (PIALES, 1996). The problem is worsened by the continued expansion of Israeli settlements and the separation wall (UAWC, 2010). To solve this problem, alternative methods for massive plant propagation like plant tissue culture techniques and other biotechnological approaches are used for producing medicinal plants, isolating medicinal secondary products, and conserving and rapid propagating of valuable, rare and endangered plant species (Julsing et al., 2007).

Capparis spinosa L., "Caper", is a well known medicinal plant in Palestine. It has many pharmaceutical activities since it contains a wide range of phytochemicals like alkaloids and flavinoids. Caper is used in our traditional medicine to treat many diseases like rheumatism and female infertility (Said et al., 2002). The conventional

propagation methods of caper by seeds and cutting are not preferred due to low germination percentage and rooting problems (Al-Safadi and Elias, 2011; Musallam et al., 2011; Rodriguez et al., 1990). *In vitro* culture of caper can solve propagation problems; it guarantees mass production of plant material without menacing natural resources and also it improves and conserves caper plant (Musallam et al., 2011). Additionally, *in vitro* culture produces disease-free plants (Al-Safadi and Elias, 2011) and provides a constant and reliable source of tissue to be used for biochemicals production.

The purpose of this study is to investigate different factors influencing the*in vitro* culture of *Capparis spinosa* L. to produce plant parts having medicinal activates in large amounts without threatening the natural resources. On other aspect, to examine the antimicrobial activities of extracts from different tissues.

1.1 Study Species

Capparis spinosa L. is one of the known medicinal plants in Palestine which has high pharmaceutical, economical and ecological values (Musallam et al, 2011). Caper belongs to the genus *Capparis* and the Capparidaceae family. The genus*Capparis* has about 350 species including shrubs, trees and woody climbers (Al-Safadi and Elias, 2011). Danin (2011) gave *Capparis spinosa* a new synonym which is *Capparis hierosolymitana* Danin or The Jerusalem Caper. *C. spinosa* is well-known with different common names such as Kaber, Âssaf, Lassaf, Tarbil, Akbaar, Shafallah, and Felfel-al-jabal (Sher and Alyemeni, 2010; Hudaib et al., 2008; Marc et al., 2008). According to Jiang et al. (2007), *C. spinosa* has a wide distribution in the Old World from South Europe, North and East Africa, Madagascar, Southwest and Central Asia to Australia and Oceania (Figure 1.1).

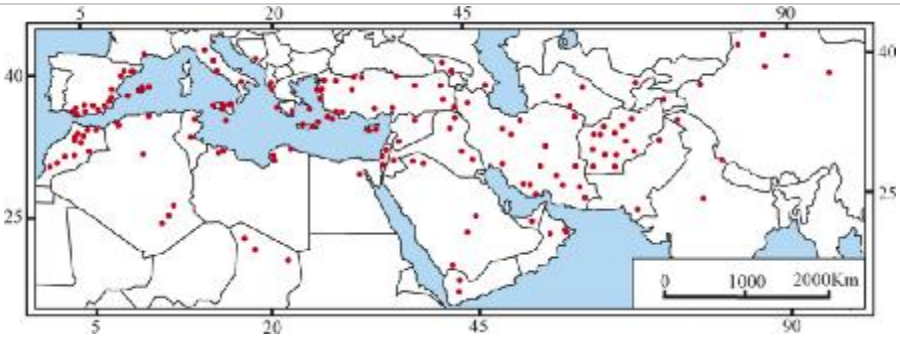

Figure 1.1: The natural distribution of caper in Eurasia and North Africa from (Jiang et al., 2007), p. 410.

Caper is a dicotyledonous, spiny medium size perennial bush. It may reach about one meter height (Basbag et al., 2009; Soyler and Khawar, 2007; Olmez et al., 2004). It grows spontaneously in cracks and crevices of rocks and stone walls. Caper adapts to poor soils where water and nutrients are major limiting factor. Also, it favors to grow in saline habitat with halophytic ecological community (Musallam et al, 2011; Sher and Alyemeni, 2010; Soyler and Khawar, 2007; Olmez et al., 2006). *C. spinosa* is a deep rooted plant; it develops large extensive root systems that penetrate deeply up to 40 m (Olmez et al., 2004). Also, it is drought tolerant and produces a great vegetative cover which protects soils from water loss and reduces soil erosion and land degradation (Sakcali et al., 2008; Soyler and Khawar, 2007; Olmez et al., 2006). The leaves of caper are alternate, opposite, simple or trifoliate, 2.0-3.0 X 1.8-3.0 cm, round to ovate, thick and glistening. Petiole is 0.5-1.0 cm long. Stipules are 0.3-0.4 cm long, spinous, stout, yellow, and hooked (Inocencio et al., 2006). Leaf stipules may be formed into spines; this is the reason why it is called "spinosa" (Basbag et al., 2009). The flower is bisexual, white fading pinkish purple and fragrant. Caper flowers from July to August. Floral pedicels are stout, 2.5-4.0 cm. Stamens are longer than petals. The plant's berry is 1.0-5.1 cm long, obovoid, red inside when ripe. The flowers produce large, kidney shaped, and gray-brown color seeds in September (Sher and Alyemeni, 2010; Satyanarayana et al., 2008).

C. spinosa has an immense number of pharmaceutical and ethnobotanical importances since it contains vital bioactive agents and has potential of producing useful biochemical compounds important for different pharmaceutical, cosmetic, and food industries.

C. spinosa is used for medicinal purposes as recorded firstly in 2000 BC by the Sumerians for treating rheumatism, anemia, and gout (Sher and Alyemeni, 2010). The ancient Greeks and Romans also used caper medicinally for expelling the tape worms when the plant mixed with vinegar and honey (Jiang et al., 2007). Around the world, caper nowadays is used in different traditional medicine, for instance, in our Palestinian folk medicine the decoction of leaves is used to treat female infertility, and the roots are applied for deafness. Stems and fruits are used for treating back diseases (Said et al., 2002). In Jordanian traditional medicine, the root bark are macerated and placed between gauze on aching area for 15-25 min in order torelief the inflammation and muscle pain (Hudaib et al., 2008). The dried fruits in Arab traditional medicines are taken orally with water for curing of hypertension and diabetes. The decoctions

from the root bark have been used to treat dropsy, anemia, and rheumatism. The tea prepared from buds and leaves is used in Saudi folklore against cold and related infections (Sher and Alyemeni, 2010). In Morocco for example, this plant is traditionally used in diabetes treatment (Lemhadri et al., 2007; Eddouks et al., 2004). Caper is used for gout, arthritic and neuralgic (sciatic and trigeminal) pains in Italy. While exclusively in Tunisia, it is employed as galactogenous (Leporatti et al., 2009). In Pakistan, the root bark is used for mental disorder, enlarged spleen and tubercular glands (Afzal et al., 2009).

Since *C. spinosa* contains a wide range of phytochemical compounds which present in various parts of the plant such as alkaloids (Capparispine), flavonoids (Rutin), lipids, polyphenols, terpenes, mustard oil glycoside, indoles and aliphatic glucosinolates (Adams et al., 2009; Rajesh et al., 2009; Wang et al., 2009; Arena et al., 2008) (Table1.1), many studies reported that different parts of caper are used to treat different problems such as rheumatism, hypertension, and diabetes (Table 1.2).Sher and Alyemeni (2010) reported that *C. spinosa* was found to be a safe plant and there are no reports available in the scientific literature on its toxic manifestations after acute, sub-acute, or chronic treatment.

Table 1.1: Some of phytochemical compounds present in different parts of *C. spinosa*.

Phytochemical Group	**Phytochemical Compound**	**References**
Spermidine Alkaloids	Capparispine, Capparispine-26-o-β-d glucoside, and Cabadicine-26-o-β-d-glucoside hydrochloride	Rajesh et al., 2009; Pu Fu et al., 2008; Satyanarayana et al., 2008.
Flavonoid	Rutin	Rajesh et al., 2009; Ramezani et al., 2008; Satyanarayana et al., 2008; Tesoriere et al., 2007.
Flavonoid	Quercetin-3-rutinoside	Satyanarayana et al., 2008; Tesoriere et al., 2007.
Flavonol Glycoside	Quercetin 3-O-[6”-α-L-rhamnosyl-6’–β-D-glucosyl]-β–glucoside	Satyanarayana et al., 2008; Sharaf et al., 2000.
Indole Glucosinolates	Glucobrassicin	Satyanarayana et al., 2008; Hamed et al., 2007.
Glycosides	1-H Indole-3-acetonitrile glycosides	Sher and Alyemeni, 2010; Rajesh et al., 2009; Arena et al., 2008; Satyanarayana et al., 2008; Calis et al., 1999.

Indole Glucosinolates	Neoglucobrassicin	Rajesh et al., 2009; Satyanarayana et al., 2008; Hamed et al., 2007.
Glucosinolates	Sinigrin	Rajesh et al., 2009; Satyanarayana et al., 2008; Hamed et al., 2007.
Triterpenodes	B –carotene	Sher and Alyemeni, 2010; Satyanarayana et al., 2008.
Hydroxy Cinnamic acids	Cinnamic acid	Satyanarayana et al., 2008; Svobodová et al., 2003.
Volatile Oils	Thymol	Rajesh et al., 2009; Satyanarayana et al., 2008.

Table 1.2: The medicinal activities of different parts of *C. spinosa*.

Plant Parts	Medicinal Activity	References
Floral buds	Anti-oxidative	Girish et al., 2009; Ramezani et al., 2008; Satyanarayana et al., 2008; Lemhadri et al., 2007; Tesoriere et al., 2007; Svobodová et al., 2003.
Root bark, Flowers, Fruits	Anti-hypertensive	Sher and Alyemeni, 2010; Tahraoui et al., 2007.
Floral buds, Roots	Arteriosclerosis	Hamed et al., 2007.
Aerial parts	Anti-hyperlipidemic	Satyanarayana et al., 2008; Tesoriere et al., 2007.
Root bark	Anti-hepatotoxic, Hepatic stimulants and protectors, Improve liver function	Sher and Alyemeni, 2010; Girish et al., 2009; Ramezani et al., 2008; Satyanarayana et al., 2008; Lemhadri et al., 2007; Eddouks et al., 2004.
Aerial parts, Fruits, Roots, Leaves	Anti-inflammatory	Sher and Alyemeni, 2010; Rajesh et al., 2009; Hudaib et al.,2008; Ramezani et al., 2008; Lemhadri et al., 2007; Tesoriere et al., 2007; Eddouks et al., 2004.
Root bark, Stems, Leaves, Fruits, Aerial parts	Rheumatism	Darwish and Aburjai, 2010; Sher and Alyemeni, 2010; Adams et al., 2009; Sahranavard et al., 2009; Marc et al., 2008; Pu Fu et al., 2008; Satyanarayana et al., 2008; Aburjai et al., 2007; Hamed et al., 2007.
Root bark, Leaves	Gout	Sher and Alyemeni, 2010; Adams et al., 2009; Marc et al., 2008; Satyanarayana et al., 2008; Hamed et al., 2007.
Fruits, Leaves	Arthritis	Adams et al., 2009; Marc et al., 2008; Aburjai et al., 2007.

Fruits	Sciatica	Marc et al., 2008.
Root bark	Analgesic	Sher and Alyemeni, 2010; Rajesh et al., 2009; Satyanarayana et al., 2008; Aburjai et al., 2007.
Fruits, Roots, Leaves	Joints and Muscle pain	Hudaib et al., 2008.
Leaves	Paralysis	Satyanarayana et al., 2008; Aburjai et al., 2007.
Aerial parts, Leaves, Root bark, Fruits	Anti-diabetic	Sher and Alyemeni, 2010; Rajesh et al., 2009; Ramezani et al., 2008; Aburjai et al., 2007; Hamed et al., 2007; Lemhadri et al., 2007; Tahraoui et al., 2007; Tesoriere et al., 2007; Eddouks et al., 2004; Jouad et al., 2001.
Fruits	Hypoglycemic	Zeggwagh et al., 2007; Eddouks et al., 2004.
Fruits	Anti-obesity	Lemhadri et al., 2007.
Leaves	Coughs	Hamed et al., 2007.
Root bark	Expectorant	Sher and Alyemeni, 2010; Rajesh et al., 2009; Satyanarayana et al., 2008; Aburjai et al., 2007; Hamed et al., 2007.
Leaves	Earache	Hamed et al., 2007.
Leaves, Roots	Toothache, Reduce swelling, Prevent dental plaque and gingival inflammation	Al-Bayaty et al., 2010; Satyanarayana et al., 2008; Hamed et al., 2007.
Bark, Floral buds	Laxative	Sher and Alyemeni, 2010; Hamed et al., 2007.
Roots	Purgative	Darwish and Aburjai, 2010.
Leaves, Floral buds	Diarrhea	Hamed et al., 2007.
Root bark, Leaves, Floral buds, Aerial parts	Gastrointestinal Problems	Sher and Alyemeni, 2010; Sahranavard et al., 2009.
Leaves, Floral buds	Antispasmodic	Sher and Alyemeni, 2010; Rajesh et al., 2009.
Root bark	Carminative	Sher and Alyemeni, 2010.
Floral buds, Roots, Stems, Leaves	Diuretic	Sher and Alyemeni, 2010; Satyanarayana et al., 2008; Aburjai et al., 2007; Hamed et al., 2007.
Root bark	Dropsy	Sher and Alyemeni, 2010.
Floral buds, Roots	Renal disinfectant	Aburjai et al., 2007; Hamed et al., 2007.
Leaves, Floral buds	Remove kidney stones	Sher and Alyemeni, 2010.
Fruits	Treat irregular	Singh and Lal, 2008.

	urination	
Floral buds, Roots	Tonic	Sher and Alyemeni, 2010; Satyanarayana et al., 2008; Hamed et al., 2007.
Root bark	Anemia	Sher and Alyemeni, 2010.
Root bark, Leaves	Anthelmintic,	Darwish and Aburjai, 2010; Sher and Alyemeni, 2010; Satyanarayana et al., 2008; Hamed et al., 2007.
Leaves	Appetizer	Sher and Alyemeni, 2010; Satyanarayana et al., 2008.
Leaves, Floral buds	Skin diseases (eczema)	Sher and Alyemeni, 2010; Aburjai et al., 2007.
Floral buds	Antiviral	Girish et al., 2009; Satyanarayana et al., 2008.
Leaves, Fruits, Root bark	Antibacterial	Bouriche et al., 2011; Boga et al., 2011; Satyanarayana et al., 2008; Mahasneh, 2002.
Aerial parts, Leaves, Fruits	Antifungal	Darwish and Aburjai, 2010; Rajesh et al., 2009; Ramezani et al., 2008; Satyanarayana et al., 2008; Lemhadri et al., 2007; Tesoriere et al., 2007; Eddouks et al., 2004; Mahasneh, 2002.
Fruits, Root bark, Leaves	Astringent	Aburjai et al., 2007.
Fruits	Cure impotency	Singh and Lal, 2008.
Aerial parts	Hemorrhoid	Sahranavard et al., 2009.

1.2 *In Vitro* Culture of *C. spinosa*

The common propagation methods of *C. spinosa* are not easy. For example, caper multiplication by seeds is unfavorable since the germination percentage is low due to seed dormancy and also the seeds have high degree of heterozygosity (Musallam et al., 2011). However, studying factors affecting seed germination is justified when addressing characters that possibly resulted from interspecific breeding or hybridization. Moreover, caper multiplication by vegetative cutting has rooting problems and low degree of success (Al-Safadi and Elias, 2011; Musallam et al., 2011; Chalak and Elbitar, 2006; Rodriguez et al., 1990). The use of *in vitro* culture technique is the best solution to overcome caper propagation problems and also it can enhance mass production of caper without threatening the natural resources. Plant tissue culture is a set of techniques for the aseptic culture of cells, tissues, and organs under defined physical and chemical conditions *in vitro* and controlled environment. The tissue culture technology explores conditions that promote cell division. Also, it

is an important tool in both basic and applied studies and for commercial applications (Kreis, 2007). *In vitro* growth and development of plant is determined by organic substance (Regulators) which are needed in very small concentrations. They control the distribution of all types of substance within plant and they are responsible for cell division and growth. Regulators (auxin and cytokinin) regulate the development of organs on parts of plants grown *in vitro.* Auxins (IAA, IBA, NAA, or 2,4-D) cause cell elongation and swelling of tissues, cell division (callus formation) and the formation of adventitious roots, while cytokinins (kinetin, BA, 2ip, or zeatin) stimulate growth and development. They enhance cell division and induce adventitious shoot formation by decreasing apical dominance (Pierik, 1987). Plant tissue culture of medicinal plants has a number of advantages as well over traditional propagation methods such as:

- The possibility of year round continuous production of medicinal plant under highly controlled conditions.
- Growing plant under *in vitro* conditions enables a constant and reliable source of tissues to be used for metabolites production; i.e. controlling physical and chemical culture conditions can guarantee a standard production of plant secondary metabolites, while *ex vitro* grown plants are exposed to all surrounding environmental conditions and their constituents may vary accordingly.
- It can eliminate concerns regarding over harvesting of rare or endangered medicinal plants (Briskin, 2007).

In the current study different *in vitro* culture techniques were used including micropropagation of caper from seeds and nodal segments. Moreover, callus and root cultures were established for secondary metabolites production and bioassay.

Seed biology is one of the most widely researched areas in plant physiology. The seed, containing the embryo as the new plant in miniature, is structurally and physiologically prepared for its role as a dispersal unit and is well provided with food reserves to maintain the growing seedling until it establishes itself as a self-sufficient, autotrophic organism. Because the function of a seed is to launch a new plant, it may seem peculiar that dormancy exists. Seed dormancy is generally an unfavorable feature in agricultural crops, where rapid germination and growth are required. However, some degree of dormancy is useful, at least during seed development. Seed

dormancy is the failure of an intact viable seed to complete germination under favorable conditions. The seeds of some species are disallowed from completing germination because the embryo is guarded by its surrounding structures. This phenomenon is known as coat enhanced dormancy; embryos isolated from these seeds are not dormant. In other species, a second category of dormancy is found in which the embryos themselves are dormant (embryo dormancy). Germination commences with the uptake of water by the quiescent dry seed (imbibition) and terminates with the elongation of the embryonic axis (radicle) extends to penetrate the structures that surround it (Derek Bewley, 1997). Seeds in many species are good starting material for *in vitro* cultures. After being surface sterilized and inoculated on proper germination media, the resulted seedlings are good source for tissues like roots, cotyledons, hypocotyls that used for the initiation of other types of cultures like callus, cells and root cultures (Kreis, 2007).

The nodal segment culture is a type of culture in which the bud is isolated with part of the stem in order to form shoot by allowing the bud to develop. This method is the most natural method of vegetative propagation of plants *in vitro* and *in vivo* (Chawla, 2007). The conventional vegetative propagation of caper has rooting problems but the micropropagation by nodal segments of caper is used to accelerate large-scale multiplication, enhance and preserve caper plant (Musallam et al, 2011).

Callus tissue is a largely unorganized, proliferating mass of parenchyma cells that is produced in response to injury. All types of organs (roots, stems, leaves, etc.) and tissues can be used to induce the growth of callus (Chawla, 2007). During callus formation there is some degree of dedifferentiation in both morphology and metabolism. One major consequence of this dedifferentiation is that most plant cultures lose the ability to carry out photosynthesis. This has important consequences for culture of callus tissue, as the metabolic profile will probably not match that of the donor plant (Bansal, 2006). The level of plant growth regulators is a major factor that affects callus formation and growth in the culture medium. The type and concentration of growth regulators depends strongly on the genotype and endogenous hormone content of explants (Chawla, 2007). After being induced, callus tissue can be routinely subcultured to a new fresh media for further growth. When subcultured regularly, callus will exhibit an S-shaped or sigmoid pattern of growth during each passage and for many species subculture periods range from three to six weeks (Chawla, 2007).

In summary, there are five phases of callus or cell culture growth (Figure 1.2):

1- Lag phase, where cells prepare to divide.
2- Exponential phase, where the rate of cell division is increasing.
3- Liner phase, where the cell division is slow but the rate of the calls expansion increases.
4- Deceleration phase, where the rate of cell division and elongation decreases.
5- Stationary phase, where the number and size of cells remain constant.

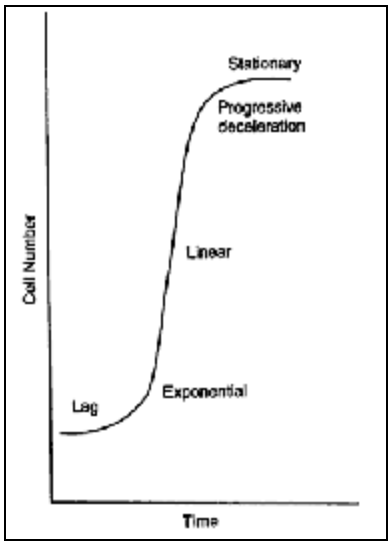

Figure 1.2: A model curve showing cell number per unit volume of culture to time in a batch grown cell suspension culture from (Chawla, 2007), p. 58.

Although all parts of caper are pharmaceutically useful, roots are the principle material used in our traditional medicine. The roots are used to treat hypertension, arteriosclerosis, gout, rheumatism, and anemia (Sher and Alyemeni, 2010; Satyanarayana et al., 2008; Hamed et al., 2007). *In vitro* root culture offers promise for high production and productivity of valuable secondary metabolites (Pandey et al., 2010) and potential for transformation by *Agrobacterium.* Hairy root cultures transformed with the pathogenic soil bacterium *Agrobacterium rhizogenes* is an attractive choice with its rapid growth and subsequent high productivity of secondary metabolites (Saito et al, 2001). They often grow faster than plant cell cultures and do not require hormones in the medium. Strain selection of good hairy root lines is important. Different strains of *Agrobacterium rhizogenes* can also affect transformation. For example, the A4 strain of *Agrobacterium rhizogenes* was more effective at inducing hairy roots from *Valeriana wallichii* D C, and had a higher

growth rate (Kim et al., 2002). Moreover, root culture grows rapidly, relatively easy to prepare and maintain, shows a low level of variability and can be simply cloned to produce a large supply of experimental tissue (Khalafalla et al., 2009; Nandagopal and Ranjitha Kumari, 2007). In present study, a reproducible method for high frequency root induction and root growth from leaf explants was established.

1.3 Extraction of Secondary Metabolites

Extraction can be defined as a method for separation of chemicals apart from plant tissues by using special solvents and techniques (Das et al., 2010; Ncube et al., 2008). The extracted product contains mixture of different plant metabolites, which can be isolated from each other by using different fractionation techniques. To increase the quality of an extract, the extraction methods, plant part used as starting material, and extraction solvents type must be taken into consideration. Many techniques are used for extraction including maceration, infusion, percolation, digestion, decoction, hot continuous extraction (soxhlet), counter current extraction (Das et al., 2010; Ncube et al., 2008). The microwave assisted extraction (MAE), supercritical fluid extraction (SFE) and accelerated solvent extraction (ASE) or pressurized liquid extraction (PLE) are new methods that are used to reduce or minimize the use of organic solvents and improve the extraction process (Mendonça-Filho, 2006). The extraction solvent must be non toxic, evaporates easily at low temperature, promote of rapid physiologic absorption of the extract, and have preservative action and inability to cause the extract to complex or dissociate (Das et al., 2010; Ncube et al., 2008). In the current study, different plant parts were extracted by an organic solvent (ethanol) and other natural solvents like apple and grape vinegars. The purpose of using natural solvents is to introduce new natural extraction solvents that can replace the organic one. These natural solvents are eco-friendly; they can eliminate the dangerous consequences of using organic solvents on environment. Also, vinegar and its natural additives may extract many compounds that cannot be extracted by organic solvent. Moreover, compounds in vinegar may conjugate selectively with bioactive compounds to generate new spectrum of drug-conjugates with improved physicochemical properties.

1.4 Antimicrobial Activity of *C. spinosa*

Nowadays, the infectious diseases are the world's leading cause of premature death which kills about 50000 people every day (Aqil et al., 2006; Eloff and McGaw, 2006).

To reduce or kill the infecting microorganisms, many types of antimicrobial agents are used. These agents have different modes of action such as inhibition of cell wall synthesis, inhibition of protein synthesis, interference with nucleic acid synthesis, injury to plasma membrane, or inhibition of a metabolic pathway (Tortora et al., 2007; Ingraham and Ingraham, 2000). The random use of antimicrobial drugs develops multi-drug resistance in human pathogens against commonly used antibiotics (Aqil et al., 2006). Accordingly, many researches have been conducted on different plants extracts to test their antimicrobial activity and to discover new antimicrobial compounds to alternate the chemical antimicrobial agents (Das et al., 2010; Maji et al., 2010; Sharma et al., 2010; Hassan et al., 2009; Ncube et al., 2008; Tortora et al., 2007; Adwan et al., 2006; C$_s$itoglu et al., 2003). The phyto-medicine succeeded in treating difficult infectious diseases including viral infections (Maji et al., 2010) because plant tissues contain secondary metabolites with antimicrobial activities like alkaloids, terpenoids, essential oils, and phenolics and polyphenols which include simple phenols, quinones, flavones, flavonoids, and tannins (Das et al., 2010; Paiva et al., 2010; Ncube et al., 2008; Cowan, 1999). Many studies scanned the antimicrobial activities of medicinal plants such as *Mentha longifolia, Melissa officinalis, Rosa damascene* (Abu-Shanab et al., 2006) and *Capparis spinosa.* For example, Boga et al. (2011) study showed that the decoction of caper roots has bacterioststic activity on the growth of *Deinococus radiophilus.*

The antimicrobial susceptibility test (AST) is widely used to evaluate the plant extracts for antimicrobial activity and to determine Minimum Inhibitory Concentration (MIC) of the antimicrobial substance (Kaçar, 2008; Ncube et al., 2008). Das et al. (2010) and Ncube et al. (2008) reported that even though the current standard AST methods accepted by different organizations like National Committee for Clinical Laboratory Science (NCCLS), British Society for Antimicrobial Chemotherapy (BSAC) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST) exist, for guidelines of antimicrobial susceptibility testing of convenient drugs, these might not be convenient to plant extracts and modifications must be made. AST standard tests are generally classified into diffusion and dilution methods. Diffusion tests include agar well diffusion, agar disk diffusion, poison food technique, and bioautography. Dilution methods include agar dilution, broth microdilution and broth macrodilution technique (Das et al., 2010; Kaçar, 2008; Ncube et al., 2008). In the present study, the agar disk diffusion assay was used to

evaluate the antimicrobial activity of*C. spinosa* extracts. This method was developed in 1940 and its procedure was accepted by NCCLS. Agar disk diffusion assay is a modification of that described by Bauer, Kirby, Sherris and Truck (commonly known as Kirby-Bauer test) (Das et al., 2010). This technique is a simple method of determining the susceptibility of a microorganism to an antimicrobial agent by inoculating an agar plate with the culture and allowing the antimicrobial agent to diffuse into the agar medium. The filter disc (6.0 mm sterilized filter papers disks (Whatmann No. 1)) that impregnated with filter sterilized plant extract of desired concentration is applied to the surface of an agar plate that containing the microorganism to be tested. The proper solid agar media are Mueller Hinton (Mueller and Hinton, 1941), Trypton soy agar or Nutrient agar. And the standard inoculum size is 1 x 10^8 CFU/ml of bacteria which is equal to McFarland 0.5 turbidity standard. Plates are then incubated for 24 hr at 37°C (bacteria) and 48 hr at 25°C (fungi). After incubation, the effectiveness of a particular antimicrobial agent is shown by the presence of growth inhibition zones. The zones of inhibition appear as clear areas surrounding the disc from which the substances with antimicrobial activity diffused. The size of the zone may be affected by the density or viscosity of the culture medium, the rate of diffusion of the antimicrobial agent, the concentration of the agent, and the sensitivity of the microorganism to the agent, and the interaction between the antimicrobial agent and the medium (Das et al., 2010; Ncube et al., 2008).

CHAPTER 2

Objectives

Since *Capparis spinosa* L. (caper) has many medicinal activities and it is still used in our traditional medicine, the aim of this study is to improve *C. spinosa* propagation and exploitation by using plant tissue culture technique and for producing tissues that have medicinal activates in large quantities without menacing the wild life and natural resources.

The specific objectives in this study are as follow:

- To investigate the chemical and physical factors those affect the *in vitro* propagation of *C. spinosa* from different explants
- To study the chemical and physical factors that influence *C. spinosa* callus induction and culture and differentiation.
- To study the chemical and physical factors that influence *C. spinosa in vitro* root induction and culture.
- To test the antimicrobial effect of *C. spinosa* extracts on different microbiological agents.

CHAPTER 3

Materials and Methods

The experimental work in this study was conducted in the Plant Tissue Culture Laboratory, Biotechnology Research Center at the Palestine Polytechnic University in Hebron, Palestine. Tests related to microbiology were conducted in Caritas Baby Hospital in Bethlehem, Palestine.

3.1 *In Vitro* Culture of *C. spinosa*

3.1.1 Sources of Plant Material

Seeds of *C. spinosa* were collected from Hebron city in September 2008 and germinated in pots and kept in the growth room for further growth. Explants like leaves and nodal segments were taken from the potted plants and used for further experiments.

Figure 3.1: Mother plants of *C. spinosa* that have been germinated from seeds.

3.1.2 Chemicals and Reagents

The basal media; MS medium (Murashige and Skoog, 1962) and Gamborgs B5 medium including vitamins (Gamborg, et al. 1968) were purchased from Duchefa biochemie, Netherlands. McCowns woody pant basal salt mixture (Mcc) Lloyd and McCown (1981) was obtained from Sigma. The carbon sources such as D-Fructose, D-Sorbitol and Sucrose were purchased from Duchefa. The plant growth regulators (PGR); 6-Benzyleaminopurine (BA), Gibberellic acid (GA_3), Indole-3-acetic acid (IAA), and 1-Naphtalene acetic acid (NAA), Zeatin, Thidiazuron (TDZ), Isopentenyl adenine (2ip) were purchased from Duchefa biochemie, whereas 2,4-Dichlorophenolxyacetic acid (2,4-D), and Indole-3-butyric acid (IBA) were purchased

from Sigma. Media was gelled by agar that was obtained from HyLabs Company for laboratory chemicals.

3.1.3 Media Preparation and Sterilization

The growth medium was prepared by using one of the basal salts of MS, B5, or Mcc. The recommended salt weight for every medium in addition to 30.0 g/L sucrose were dissolved in distilled water up to 50 % of the final volume. The needed plant growth regulators (PGR) were added then the media was brought to the final volume. Medium pH was adjusted to 5.8 by pH meter with 1.0 M KOH or 1.0 M HCl. Media was solidified with 6.0 g/L agar. All components were heated with continuous stirring until they completely dissolved. Finally, media were dispensed in autoclavable media bottles for use in sterile polystyrene Petri dishes. Media in other experiments including nodal segments culture and root culture was poured in 16x160 mm test tubes (8.0 ml) or in 50.0 ml Erlenmeyer flasks (8.0 ml). Media autoclaved at 121°C and 15 of Psi pressure for 20 min.

3.1.4 Sterilization of Plant Material

Explants like seeds, leaves, and nodal segments were surface sterilized by washing thoroughly under running tap water for 30 min then soaked in water and commercial detergent for 1.0 hr with continuous agitation. In the laminar flow cabinet, leaves and nodal segments were immersed in 100 ml of 15% v/v Chlorex (5.0 % sodium hypochlorite) for 10 min with continuous shaking on orbital shaker at 110 rpm. For starting *in vitro* seed culture, seeds were dipped in 100 ml of 20 % v/v Chlorex for 30 min. Chlorex was removed by rinsing explants with sterile distilled water (SDW) three times two min each. Finally, explants were dipped in 70 % ethanol for 30 sec then washed by SDW twice 2.0 min each.

3.1.5 Growth Conditions

Cultures were incubated in the growth room at 24±1ºC with 16:8 light:dark photoperiod under cool-white fluorescent illumination of 40-45 µmol/m^2/sec photosynthetic photon flux density (PPFD).

3.1.6 Experimental Design and Statistical Analysis

All experiments were designed in a Completely Randomized Design (CRD) and significance in data means was tested by the analysis of variance ANOVA with StatPlus 2007 Professional software. In the case of Significant ANOVA at p=0.05, means were separated by Fisher LSD test. Alternatively, when comparing between two means, statistical T-test was conducted at p=0.05. Data with unequal variance or not normally distributed, the non-parametric test of Kruskal-Wallis ANOVA was carried out.

3.1.7 Culture Types

3.1.7.1 *In Vitro* Seed Germination

It is documented that *C. spinosa* seeds exert at least one type of dormancy; for example, Pascual et al. (2006) addressed the effect of the duration of the seed storage period on seed germination. Soyler and Khawar (2007) and Olmez et al. (2004) studied the factors affecting seed germination after scarification and sulfuric acid pretreatment. Basbag et al. (2009) investigated the effect of different temperatures on seed germination. In this study, the following three approaches were conducted to investigate the factors that would promote seed germination and overcome seed dormancy:

1. Effect of basal salt media: MS and Mcc media supplemented with 0.0, 1.0, and 2.0 mg/L GA_3 were cultured by newly harvested seeds under light and dark conditions. Seeds were soaked in 5.0 ml solution containing 5.0 mg/L GA_3 for 72 hr at 4°C.
2. Scarification of seed coat: the seed coat of newly harvested sterilized seeds was cut with a blade in the Laminar Flow cabinet and the seeds were cultured on the same media in pervious experiment and kept under light. Seeds were soaked in 5.0 ml solution containing 5.0 mg/L GA_3 for 72 hr at 4°C.

Seeds in these two treatments were cultured on 5.0 cm Petri-dishes that were filled with 8.0 ml of media. Five replicates with five seeds per replicate were used in each media.

3. Effect of seed age, cold pretreatment, and GA_3 treatment: freshly collected seeds, one year old seeds and two years old seeds were used to examine their germinability in four different treatments:

a. Cold treatment: using seeds that were stored in 4°C for four months.
b. GA_3 treatment: using seeds that were soaked in 5.0 ml solution containing 5.0 mg/L GA_3 for 72 hr.
c. Cold and GA_3 treatments: using seeds which were stored at 4°C for four months and then the seeds were soaked in 5.0 ml solution containing 5.0 mg/L GA_3 for 72 hr at 4°C.
d. Untreated seeds: seeds were cultured without any pretreatment.

The sterilized seeds were cultured on full strength MS medium supplemented with 1.0 mg/L GA_3. Three replicates with five seeds per replicate were used in each treatment and the plates were kept in growth room under light condition.

3.1.7.1.1 Subculture of *In Vitro* Germinated Seeds

The germinated seeds were transferred and cultured in plastic pots containing sterilized peatmoss. Transferred plants were monitored after two and four weeks to evaluate their growth performance.

3.1.7.2 Shoot Proliferation and Regeneration from Nodal Segments

To examine the optimum medium for shoot proliferation, nodal segments were excised from the mother plant and surface sterilized. They were cultured in test tubes filled with the following media:

a. Full strength MS medium with 0.5 mg/L IAA, 1.0 mg/L GA_3, and different BA concentrations (0.0, 0.5, 1.0, 1.5, and 2.0 mg/L).
b. Full strength MS medium containing zeatin at different concentrations (0.0, 0.5, 1.0, and 2.0 mg/L).

Each treatment composed of ten tubes as replicates.

For further growth of the regenerated plantlets, they were subcultured three times in MS media supplemented with 1.0 mg/L zeatin.

3.1.7.3 Callus Induction and Culture

3.1.7.3.1 Callus Induction

Callus was induced from leaf discs. The sterilized leaves were cut into 5.0 mm discs and inoculated on 5.0 cm Petri-dishes filled with 8.0 ml of the needed media. Each Petri-dish was cultured by four explants.

To examine whether light affects callus induction, sterile leaf discs were placed on full strength MS medium that supplemented with 0.5 mg/L 2,4-D. Six Petri-dishes were kept under dark and the other six under light conditions in the growth room. Depending on the results of this treatment, next experiments were conducted.

In order to study the effect of basal media on callus induction, the sterile leaf discs were cultured on MS and B5 media each was supplemented with 0.5 mg/L 2,4-D. Three Petri-dishes from each treatment were kept in the growth room in the dark cabinet.

To evaluate the effect of 2,4-D concentration on callus induction, the PGR was added at 0.0, 0.1 and 0.5 mg/L to full strength MS media. Three Petri-dishes of each treatment were used.

To generate the growth curve for callus induction, four sterilized leaves were cultured on full strength MS medium supplemented with 0.5 mg/L 2,4-D in 15 Petri-dishes. Callus fresh weight in three plates was evaluated weekly.

3.1.7.3.2 Callus Growth and Subculture

To determine the optimum medium for callus growth, induced callus clumbs (1.0 cm diam.) were subcultured on full strength MS supplemented with different concentrations and combinations of 2,4-D and BA (Table 3.1). Three Petri-dishes from each medium were cultured and kept in dark.

Table 3.1: Treatments that were used for testing callus growth.

Treatment No.	2,4-D (mg/L)	BA (mg/L)
1	0.5	0.0
2	1.0	0.0
3	0.5	1.0
4	1.0	1.0
5	1.0	1.5

To test the effect of different cytokinin types together with light or dark on callus growth, 1.5 mg/L of different PGR types including Zeatin, TDZ, 2ip, and BA were used in full strength MS media and 1.0 mg/L 2,4-D. The statistical design was a 4×2 factorial (four PGR types and two light conditions), eight Petri dishes in each treatment were used where four were kept under light and the other four were kept in the dark cabinet. The average starting weight of callus (inoculum) was 46.50 mg and data was recorded after four weeks.

3.1.7.4 *In Vitro* Root Culture

3.1.7.4.1 *De Novo* Root Induction from Leaf Disc

Roots were induced from sterilized leaf segments (0.8 cm× 0.6 cm) on 5.0 cm Petri-dishes filled with 8.0 ml of full strength MS media according to the following treatments:

a. Treatment with NAA, IBA or IAA at the concentrations of 0.0, 0.1, 0.5, and 1.0 mg/L each.
b. Different combinations and concentration of auxins (Table 3.2).
c. Different carbon sources; sucrose, fructose and sorbitol with different concentrations in combination with 1.5 mg/L NAA and 7.0 g/L agar (Table 3.3).
d. NAA at 1.0 mg/L cultured by leaf explants obtained from two different sources; potted and *in vitro* grown plants.

Experiments were carried out by placing the abaxial (lower) surface of the explants facing the medium. For each different treatment, four explants were cultured on dark condition in growth room. In treatment (a), four Petri dishes were kept in dark and the other four under light condition. In treatment (c), three Petri dishes were used. Data were reported after six weeks of culture.

Table 3.2: Media types which were used for root induction that consisted of different combinations and concentrations of the auxins NAA, 2,4-D, IBA, and IAA.

Medium No.	NAA (mg/L)	2,4-D (mg/L)	IBA (mg/L)	IAA (mg/L)
1	1.0	0.1	0	0
2	1.0	0.5	0	0
3	1.0	1.0	0	0
4	1.0	1.5	0	0
5	2.0	1.0	0	0
6	2.0	1.5	0	0
7	1.0	0	0.1	0
8	1.0	0	0.5	0
9	1.0	0	1.0	0
10	1.0	0	1.5	0
11	2.0	0	1.0	0
12	2.0	0	1.5	0
13	1.0	0	0	0.1
14	1.0	0	0	0.5

Table 3.3: Different sugar types and levels used in MS media supplemented with 1.5 mg/L NAA for root induction.

Medium No.	Sucrose (g/L)	Fructose (g/L)	Sorbitol (g/L)
1	0.0	0.0	0.0
2	30	0.0	0.0
3	15	0.0	0.0
4	0.0	30	0.0
5	0.0	15	0.0
6	0.0	0.0	30
7	0.0	0.0	15

3.1.7.4.2 Root Culture and Growth

3.1.7.4.2.1 Root Growth on Semi-solid Media

The root growth was investigated by culturing the leaf segments with induced roots on full strength MS media supplemented with

a. Different concentrations and types of auxins (Table 3.4).
b. Two different NAA levels (1.0 and 2.0 mg/L) and leaf segments from two different sources; potted and *in vitro* grown plants.

The media were solidified by 2.5 g/L agar. Three replicates of 5.0 cm plats for each treatment were used and each one had four explants. The Petri dishes were kept in the dark cabinet. Data were reported after six weeks of culture and root growth was determined by measuring the roots growth area in cm^2.

Table 3.4: Semi-solid media that contained different concentrations and types of auxins which used for root growth.

Medium No.	NAA (mg/L)	IBA (mg/L)
1	0	0
2	1.0	0
3	2.0	0
4	1.0	1.0
5	2.0	1.0

3.1.7.4.2.2 Root Growth on Liquid MS Media

The root growth was investigated by culturing the leaf segments with the induced roots in 8.0 ml of full strength liquid MS media supplemented with NAA at 1.0 or 2.0 mg/L in 50 ml Erlenmeyer flasks. The cultures were kept under continuous agitation at 80 rpm on a rotary shaker and covered with aluminum foil to ensure darkness. Six replicates for each treatment were evaluated. Regular measurement of root growth in different treatments was assessed in terms of root growth area at two weeks interval. The root growth area was determined up to a period of eight weeks.

3.2 Extraction of Secondary Metabolites

3.2.1 Plant Material

Roots of *C. spinosa* L. were collected from Taffuh village, West of Hebron. The root bark was isolated from wood, cleaned with wet tissue paper to remove any debris and dried at room temperature in shade. The dried root bark was stored in glass container until the time of extraction. The dried root bark was powdered by a blender.

Leaves of *C. spinosa* were collected from Halhol village, North of Hebron. The leaves were detached from branches and rinsed with tap water to remove the dirt, then were air-dried in shade at room temperature. The dried leaves were stored in glass container until the extraction step. The dried leaves were powdered at the extraction day.

Callus that was cultured in dark on MS medium supplemented with 1.0 mg/L 2,4-D and 1.5 mg/L BA was dried at room temperature in shade and powdered by mortar and pestle. Additionally, fresh callus was also used for extraction.

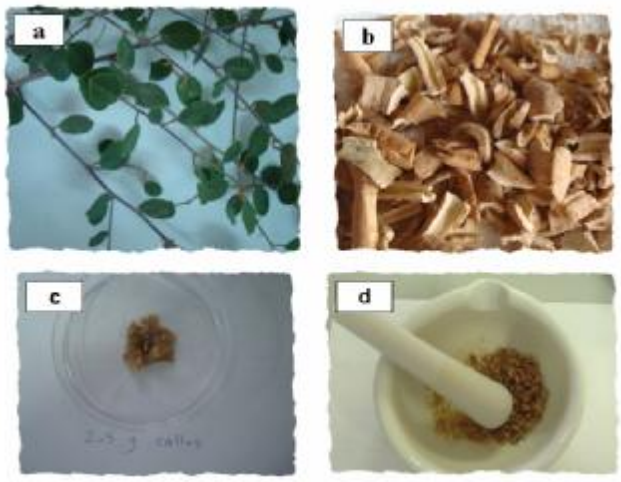

Figure 3.2: *C. spinosa* plant materials which were used in extraction. (a) leaves, (b) root bark, (c) fresh and (d) dried callus.

3.2.2 Chemicals and Reagents

For extraction, absolute ethyl alcohol, methyl alcohol, chloroform, and glacial acetic acid were purchased from Frutarom chemical company. Dimethylsulfoxide (DMSO) was purchased from aMResco chemical company. Tris base was purchased from Promega. Natural liquids as grape and apple vinegars were homemade.

3.2.3 Extraction Procedure

3.2.3.1 Extraction with Chemical Solvents

The powdered plant materials, root bark, leaves, and dry callus were dissolved in absolute ethyl alcohol at 1:100 (w/v). The mixture was placed in water bath at 99°C for 30 min. In order to replace the evaporated alcohol, the same volume of ethyl alcohol were boiled in water bath until reaching to 78°C (the boiling point of ethyl alcohol is 78.4°C) and then were added to the flask with mixing. When the flask is cold, it was closed by Parafilm and covered with aluminum foil and then kept on magnetic stirrer overnight. In the next day, the mixture was centrifuged at 5000 rpm for 15 min and the supernatant was taken and placed in falcon tubes and kept opened in the hood to evaporate the solvent. The dried extract was stored at -20 °C after ethyl alcohol was completely evaporated.

3.2.3.2 Extraction with Natural Solvents

The powdered *C. spinosa* plant materials including root bark, leaves, and dry and fresh callus were mixed with apple vinegar and grape vinegar in two separated experiments according to the amounts in Table 3.5. The mixtures were placed on magnetic stirrer and covered with aluminum foil. After 12 days, the mixture was centrifuged at 5000 rpm for 15 min and the supernatant was taken and poured in glass plate. The remaining precipitates were dissolved by few drops 3.0% acetic acid and centrifuged again. The supernatant was taken and placed in glass plate and then kept in hood to evaporate the solvents. Two days later, the solvents were evaporated and a gummy material like honey is obtained. The color of the material which was obtained from apple vinegar was dark brown, whereas the one which obtained from grape vinegar was pale yellow. The gummy material was collected in 50 ml falcon tube. The bound substances in the plate were dissolved by few drops of methanol and collected in falcon tube. The falcons were covered by aluminum foil and kept opened in hood to evaporate the solvents. After 23 days, the extracts were completely dried and kept in -20°C.

Table 3.5: Plant material weight and natural solvents volume were used in extraction procedure.

Plant Material Weight (g)	Grape Vinegar Volume (ml)	Apple Vinegar Volume (ml)
Dry root bark (2.5)	250	250
Dry leaves (2.5)	250	250
Fresh callus (2.5)	250	250
Dry callus (1.0)	100	100

3.2.4 Determination of Total Soluble Solid (TSS) in Vinegar

To determine the extract yield, pure vinegar weight is determined and subtracted. Sample of grape vinegar was centrifuged at 5000 rpm for 15 min and 10 ml of the supernatant were placed in glass plate with known weight. Five plates were used and the plates were kept under hood for drying. The weight of plates was taken every day until constant weight is achieved. The average of plate weights was calculated and the weight of vinegar per 1.0 ml was obtained. The same procedure was repeated with apple vinegar.

3.2.5 Evaluation of Vinegar Solubility

To test the efficiency of vinegar to dissolve natural product, the precipitate that obtained after centrifugation of dry callus apple vinegar extract was divided into three parts; the first part was dissolved in 50 ml distilled water, the second and third parts were dissolved in 50 ml chloroform, and 50 ml (95%) ethyl alcohol respectively. The solutions were covered by aluminum foil and mixed from time to time. After two weeks, the falcons were centrifuged at 5000 rpm for 15 min and the supernatant was taken into glass plate and kept under hood for drying. Two days later, the extracts were dried completely and the weights of plates were taken.

3.2.6 Spectrophotometric Quantification of Vinegar Extracts

The blanking of spectrophotometer was done in 700 µl DMSO. Then, 31.0 mg of pure apple vinegar were dissolved in 700 µl DMSO (44 mg/ml), the spectrum of pure vinegar was measured. Similarly, spectra were measured for apple and grape extract vinegars.

3.3 Antimicrobial Activities of *C. spinosa* Extracts

3.3.1 Determination of the Relative Antibacterial Activities of *C. spinosa* Extracts by Disc Diffusion Assays

Disc diffusion Susceptibility Testing (Kirby-Bauer, Method) was performed to screen of relative antimicrobial effects of *C. spinosa* extracts from different plant sources (root bark, leaves, and callus). The procedure used in this assay is a modification of the Kirby-Bauer test which is commonly used as an antimicrobial susceptibility testing. The detail procedure of the disc diffusion assay was expressed in Appendix B.

3.3.1.1 Microbial Cultures and Growth Conditions

In the disc diffusion assays, three bacterial strains and two fungal species were chosen to test the antimicrobial activities of *C. spinosa* extracts. *Escherichia coli; E. coli,* strain (001RH) "a kind gift from Mr. Mohammad Qabajah, Palestine Polytechnic University, mohammad_qabaja@ppu.edu'. This *E. coli* strain (001 RH) is resistant to four antibiotics; tetracycline, ciprofloxacin, kanamycin, and chloramphenicol. Moreover, the strain is sensitive to other antibiotics like ampicillin, amoxicillin, neomycin, gentamicin, nitrofurantoin, and cephalexin. *Staphylococcus aureus;* methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) and fungal species; *Candida albicans* and *Candida parapsilosis* were obtained from Caritas Baby Hospital.

Cultures of *E. coli* bacteria were grown on MacConkey Agar media (OXOID) and *S. aureus* were grown in Sheep Blood Agar media (OXOID) for 18 hr at 37°C. The bacterial species were maintained on Mueller-Hinton Agar (OXOID) at 37°C. Cultures of fungi including *Candida albicans* and *Candida parapsilosis* were grown in Sheep Blood Agar (OXOID) and were maintained on Potato Dextrose Agar (Neogen) at 37°C for 48 hr.

3.3.1.2 Determination of the Microbial Load in Assays

For result accuracy, the same microbial load each time that is examined in the antimicrobial susceptibility tests (AST). Incubation times before the inoculation with microorganisms are changeable for each strain, but it is necessary to inoculate them when microorganisms are in their logarithmic phases. In this study for adjusting the microbial load the incubation and inoculation procedures were kept the same and microbial loads were confirmed each time by standardization of inoculum size which is equal to McFarland 0.5 turbidity standard for bacteria (1.0-2.0 x 10^8 CFU/ml) and McFarland 1.0 turbidity standard for fungi (3.0 x 10^8 CFU/ml)

CHAPTER 4

Results and Discussion

4.1 *In Vitro* Culture of *C. spinosa*

4.1.1 *In Vitro* Seed Germination

In the present study, different procedures were carried out in order to overcome the seed dormancy and increase seed germination percentage. The newly harvested caper seeds were soaked in 5.0 mg/L GA_3 for 72 hr at 4°C, and then were cultured on different media types (Table 4.1). Germination results were observed after two months of culture. The germination percentages under dark and light conditions are presented in table 4.1.

Table 4.1: The seeds germination percentages of newly harvested seeds as observed in different media types under light and dark conditions after two months of culture. Sample size (n) = 25.

Medium Type	Germination Percentage (%)	
	Under Light Condition	Under Dark Condition
Free MS	8	4
MS + 1.0 mg/L GA_3	4	4
MS + 2.0 mg/L GA_3	8	4
Free Mcc	0	0
Mcc + 1.0 mg/L GA_3	4	0
Mcc + 2.0 mg/L GA_3	4	0

Low seed germination was observed ≤ 8% in all media types and under dark or light conditions. In all tested media, seeds that were cultured under light gave higher germination percentage compared to those that were cultured under dark condition. On the other hand, regardless to the light conditions, MS media supplemented with GA_3 at different levels (0.0, 1.0, or 2.0) gave higher germination percentage compared to the same levels added to Mcc media (Table 4.1). No seed germination was observed on Mcc media supplemented with different GA_3 levels (0.0, 1.0, and 2.0) and cultured under dark condition.

In another experiment, scarification of seed coat resulted in slightly increased germination percentage in all media used (Table 4.2) with no more than 12% in the case of MS hormone-free medium. This demonstrates the existence of another type of dormancy rather than physical or seed coat imposed dormancy.

Table 4.2: The percentage of germination after seed scarification. The newly harvested seeds were cultured for two months under light condition on different media types. Sample size (n) = 25.

Medium Type	Germination Percentage (%)	
	Untreated Seed coat	Cut seed coat
Free MS	8	12
MS + 1.0 mg/L GA_3	4	8
MS + 2.0 mg/L GA_3	8	8
Free Mcc	0	0
Mcc + 1.0 mg/L GA_3	0	4
Mcc + 2.0 mg/L GA_3	4	4

The influence of multifactors including seed age, cold pre-treatment (at 4°C for 16 weeks) and GA_3 treatment on germination was observed after two months of seed culture on MS medium supplemented with 1.0 mg/L GA_3. Results indicate that 40% of the two years old seeds that were treated by GA_3 have germinated. On the other hand, 33.3% of the two years old seeds that were stratified and pretreated with GA_3 have germinated. One year old seeds that were treated by GA_3 gave only 6.7% germination and no seed germination was reported in other treatments. In case of newly harvested seeds, no seeds germination was observed in any of the treatments (Table 4.3).

Table 4.3: Germination percentage of seeds with different ages (0.0, 1.0, and 2.0 years old). Seeds were cultured on MS medium supplemented with 1.0 mg/L GA_3 after two months of culture under light condition. Sample size (n) =15.

Treatment Type	Seed Germination Percentage (%)		
	Two Years Old	One Year Old	Newly Collected
Cold	0	0	0
GA_3	40	6.7	0
Cold and GA_3	33.3	0	0
No Treatment	0	0	0

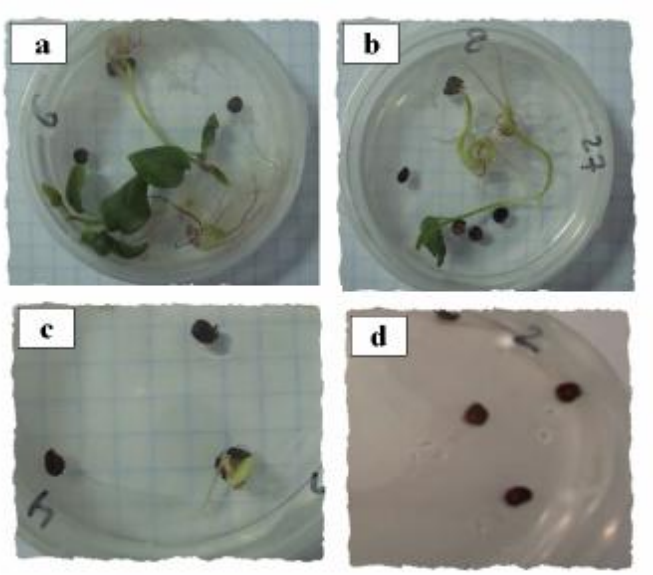

Figure 4.1: Seed germination after two months of culture with different seed pretreatments and ages. (a) seed germination of two years old seeds that were stratified and soaked in GA_3, (b) seed germination of two years old seeds were pretreated with GA_3 only, (c) seed germination in one year old seeds that stratified and soaked in GA_3, (d) no seed germination on one year old seeds were pretreated with cold and GA_3.

Under natural conditions, propagation of *C. spinosa* is complicated by limited and uneven seed germination due to seed coat imposed dormancy (Soyler and Khawar, 2007; Piotto et al., 2003). Caper seeds have impermeable hard seed coat (physical dormancy) that hinder moisture uptake (Piotto et al., 2003). Additionally, the mucilage layer that formed when the seeds placed in water makes an efficient barrier against the diffusion of oxygen to the embryo (Soyler and Khawar, 2007; Olmez et al., 2006). Therefore, many treatments such as stratification or high temperature treatment, light, pre soaking in growth regulators and partial or complete removal of the seed coat were evaluated to improve seed germination results (Basbag et al., 2009; Soyler and Khawar, 2007, Olmez et al., 2004). Moreover, Soyler and Khawar, (2007) showed that caper seeds pretreated with plant growth regulators like GA_3 or NAA influenced seed germination. In the aforementioned studies, no clear evidence about single factor might influence seed germination. Soyler and Khawar (2007) and Piotto et al. (2003) reported that the type of caper seed dormancy is seed coat imposed dormancy. They studied the effect of scarification with sulfuric acid and GA_3 treatment on germination. They reported that seed pretreatment increased the germination of 61% and 70% Soyler and Khawar (2007) and Piotto et al. (2003) studies respectively. Moreover, Piotto et al. (2003) revealed that partial or total removal of the seed coat resulted in very high percentages of germination and this

proves the essential role of seed coat in the caper's germination. However, Pascual et al. (2006) addressed the effect of the duration of the seed storage period on caper seed germination. They reported that the germination percentage was affected by both storage period and pretreatment with sulfuric acid followed by GA_3 pretreatment.

Few studies addressed establishment of *in vitro* culture of *C. spinosa* from seeds, a recent study by Al-Safadi and Elias (2011) evaluated different treatments to break caper seed dormancy; they tried seed coat scratching by iron, irradiating the seeds with gamma radiation, acid treatment by H_2SO_4, scratching and acid treatment, and treatment with ultrasonic waves. Results showed that gamma irradiation gave a significant effect on breaking dormancy and germination. Percentage of *in vitro* seed germination reached 50% with 100 Gy dose compared to 0.0% in the control treatment. On the other hand, the stimulation of caper seed germination at 100 Gy dose reached 70% compared to only 5% in the control when the seed were planted in peatmoss. Moreover, they reported that treating caper seed with sulfuric acid for 20 min combined with scarification prior to planting was effective in inducing the dormant seed to germinate where 47% of the seed germinated in comparison to no germination in the control. Furthermore, Percentage of seed germination reached 32% when the seeds were treated with only sulfuric acid and only 20% when they were scratched without acid treatment.

In this study, more chemical and physical factors were evaluated. The results show low seed germination (≤ 8%) in all media types and under dark or light conditions. Moreover, scarification of seed coat resulted in low (≤ 12%) germination percentage in all media used. In these experiments, neither GA_3 treatment nor cold pretreatment have influenced seed germination. Moreover, the age of seeds is observed to have an important role on induction of seed germination. The seeds become gradually non dormant due to after-ripening period in dry stored seeds. The storage time required differs from species to species and it was thought that dry storage alters the properties of seed coat and the endogenous hormonal level so that germination becomes possible (Dasti et al., 2001). In the present study, the storage time of caper seeds together with treatment have an important role in germination. Results show that seeds treated with GA_3 gave 40% germination when they are two years old and the percentage dropped to 6.7% in one year old seeds. No seed germination is observed with newly harvested seeds. Accordingly, the duration of caper seed storage is an important factor for

promoting seed germination, and the similar findings were reported by Dasti et al. (2001). They showed that freshly harvested *Arabidopsis thaliana* seeds were dormant but gradually became non-dormant with increased dry storage duration and this demonstrates that dormancy overcame spontaneously with time. On the other hand, Pascual et al. (2006) showed that the germination percentage of *C. spinosa* cv. *Comun* from Spain was affected by both storage period and pretreatment. The germination percentage of recently harvested control seeds was low (30%) and the same values were obtained with seeds stored up to three years. Four years stored seeds gave 18%. However, the treated seeds (scarification followed by addition of GA_3) had a high germination percentage; 89% with recently harvested seeds, 85% with one year stored seeds, and 80% with two years stored seeds. The germination percentage decreased significantly to 51% after three years and to 35% after four years of storage. These results disagree with the present results; in which the storage duration increased the germination percentage. This contradiction can be attributed to the difference in ecotype and culturing conditions (*ex* or *in vitro*). Moreover, the present study results are in disagreement with Rice et al. (2001) observation in which the four years old seeds of *Bromus tectorum* were germinated slowly with low germination percentage compared to new seeds. Furthermore, Badoni et al. (2009) showed that old seeds of *Rheum emodi* were not germinated even it was pretreated by growth regulators whereas the new seeds which were treated with GA_3 gave 25 % germination percentage.

In *ex vitro* conditions, many studied were conducted and resulted in high caper seed germination compared to that conducted under *in vitro* condition. For example, Piotto et al. (2003) achieved 70% of germination in *C. spinosa* when the seed were soaked for 1.0-1.5 hr in a solution of 400 mg/L GA_3 after the seeds chemical scarification with sulphuric acid for 15-30 min. Moreover, in Olmez et al. (2004) study, the highest germination percentage was 27.4% in seeds soaked in 300 mg/L GA_3 for 3.0 hr after treatment with H_2SO_4 for 30 min. Soyler and Khawar (2007) found that the use of a high concentration (2000 mg/L) GA_3 after immersing the seeds in warm water and treating them with sulphuric acid for 20 min resulted in seed germination of 61% at any duration compared to any other concentration of GA_3. In these previous studies, the researchers used high concentration of GA_3 after treating the seeds with H_2SO_4; i.e. GA_3 has a secondary synergic effect on germination. Moreover, the study of Pascual et al. (2006) showed that seeds treatment with acid scarification alone

reduced both viability and germination percentage, while adding GA_3 increased germination percentage without affecting viability. In the current study, GA_3 was used alone without scarification with H_2SO_4 and this justified the low seed germination percentage.

Many studies showed the important effect of cold stratification treatments on braking seed dormancy (Basbag et al., 2009; Olmez et al., 2006). In the current study, cold treatment had not any effect on seed germination when used alone without the help of GA_3 and consideration of the seed age (Table 4.3); no seed germination was observed in cold treated seeds, while 33.3% seed germination was observed in two years old seeds which were treated by cold and GA_3. These results are in disagreement with Basbag et al (2009) results which showed that the different temperature treatments were effective on mean germination percentage and the highest percentages of mean germination were obtained at 0^oC with 29.52% and the lowest percentages of mean germination were observed at control seeds with 8.39%. Additionally, Olmez (2006) showed that the duration of cold stratification was positively effective on germination percentage of the seeds, and the highest germination percentage (46.6%) was obtained in seeds that were cold stratified for 60 days, the lowest germination percentage (3.67%) was determined in control seeds.

4.1.1.1 Culture of Germinated Seeds and Acclimatization

The germinated seeds were transferred into plastic packs containing sterile peatmoss. To maintain high relative humidity, packs were placed in closed plastic bags. Relative humidity was reduced gradually and complete removal of the plastic bags took place after two weeks of placement (Figure 4.2).

Figure 4.2: Germinated seed (a) is cultured in MS medium supplemented with 1.0 mg/L GA_3, (b) *C. spinosa* seedling planted in peatmoss filled pack.

4.1.2 Shoot Proliferation and Regeneration from Nodal Segments

The effect of different media on shoot induction, elongation and plant proliferation was evaluated by measuring the shoot height, number of shoots, and number of leaves after four weeks of culture. Table 4.4 shows the effect of different levels of BA added to MS media with 0.5 mg/L IAA and 1.0 mg/L GA_3 on shoot multiplication.

Table 4.4: Effect of different levels of BA supplemented to MS media containing 0.5 mg/L IAA and 1.0 mg/L GA_3 on shoot induction, elongation and plant proliferation of *Capparis spinosa* after four weeks of culture. Sample size (n) = 10.

Concentration of BA (mg/L)	**Number of Leaves ± SE**	**Shoot Height (cm) ± SE**	**Number of Shoots± SE**
MS Free (control)	2.4 ± 0.72	0.92 ± 0.26	1.00 ± 0.00
0	2.6 ± 0.90	0.95 ± 0.33	1.10 ± 0.10
0.5	4.2 ± 1.74	0.46 ± 0.21	1.30 ± 0.15
1.0	3.1 ± 1.34	0.32 ± 0.12	1.20 ± 0.13
1.5	1.9 ± 1.27	0.20 ± 0.13	1.10 ± 0.10
2.0	2.5 ± 1.29	0.19 ± 0.12	1.20 ± 0.13

According to the number of leaves and shoot height, the significance between treatments was tested by Kruskal-Wallis ANOVA since the data is not normally distributed. Results show no statistically significant differences ($p > 0.05$) between the six tested media. MS medium with 0.5 mg/L BA was the optimum medium for plant proliferation than the five other media. The mean number of leaves in this medium was 4.2 ± 1.74 and the mean number of shoots was 1.30 ± 0.15. However, MS medium with 0.0 mg/L BA gave the highest shoot (0.95 cm± 0.33) compared to the other treatments.

Many studies showed that BA has a significant effect on shoot multiplication. For example, Rodriguez et al. (1990) reported that BA proved to be an efficient proliferating agent in all concentrations assayed by stimulating specific rates of simultaneous multiple shoot growth of caper. A similar observation was noted by Daffalla et al. (2011); they reported that BA seemed to be maintaining sufficient contents of phytohormones important for efficient organogenesis, proliferation, and shoot development and was not associated with callus induction in *Boscia senegalensis* (Capparidaceae). Furthermore, Abbas and Qaiser (2010) results showed that different concentrations of BA in MS medium had significant effect on shoot

regeneration frequency (65.00%) and number of shoots per explant in *Cadaba heterotricha* (Capparaceae). In the present study, it is concluded that the increasing level of BA has inhibitory effect on shoot height, however, increasing BA level increased the number of leaves and number of shoots at 0.5 mg/L while decreased the number of leaves, shoot height, and number of shoots when increased (Table 4.4). According to the shoot height, the present result is compatible with Rodriguez et al. (1990) and Musallam et al. (2011) results. They showed that increasing BA levels decreased caper shoot height. However, according to the number of shoots, the current result is disagreed with Rodriguez et al. (1990) and Musallam et al. (2011) results in which the number of shoots increased when BA levels increased. Moreover, Daffalla et al. (2011) reported that increasing BA levels increased the number of shoots and decreased shoot length of *Boscia senegalensis* (Capparidaceae). On the other hand, Chalak and Elbitar (2006) study showed that changing BA level from 1.0 to 2.0 mg/L did not improve significant caper shoot proliferation and plant height.

In the current study, the results show that MS medium with 0.5 mg/L BA produced the highest mean number of leaves and shoots (4.2 ± 1.74 and 1.30 ± 0.15) respectively. This result differed with the result of Rodriguez et al. (1990); they reported that 2.0 µM (= 0.45 mg/L) of BA induced poor proliferating but stimulate elongation, while 4.0 µM (= 0.9 mg/L) BA was the optimum concentration because it facilitated a high rate of proliferation and shoot development and also no callus was formed. In the present investigation, higher BA concentrations (1.5 and 2.0 mg/L) resulted in low number of leaves and shoot length. This result is in accordance with Rodriguez et al. (1990) result. They showed that the higher concentration of BA (1.35 and 1.8 mg/L) reduced shoot height.

The effect of different concentrations of zeatin on shoot induction, elongation and plant proliferation was evaluated and the data is shown in Table 4.5 and Figure 4.3.

Table 4.5: Effect of different concentrations of zeatin supplemented to MS media on shoot induction, elongation and plant proliferation of *Capparis spinosa* after four weeks of culture. Sample size (n) = 10.

Concentration of Zeatin (mg/L)	**Number of Leaves ± SE**	**Shoot Height (cm) ± SE**	**Number of Shoots ± SE**	**Callusing Percentage (%)**
0.0	4.3 ± 0.62	1.22 ± 0.15	1.10 ± 0.10	30.00
0.5	6.6 ± 1.28	1.24 ± 0.11	1.50 ± 0.22	0
1.0	3.9 ± 1.05	0.84 ± 0.21	0.80 ± 0.13	10.00
2.0	5.0 ± 0.99	1.09 ± 0.17	0.90 ± 0.10	10.00

According to the number of leaves, shoot height, and the number of shoots, the significance between treatments was tested with analysis of variance by One-Way ANOVA. Results show no statistically significant differences ($p > 0.05$) between the four tested media. Despite that the ANOVA analysis resulted in non significant difference in the three evaluated parameters, zeatin at 0.5 mg/L gave highest number of leaves (6.6 ± 1.28), shoot height (1.24 cm ± 0.11), and number of shoots (1.5 ± 0.22) compared to other concentrations. Moreover, no callus was formed at this level. In this study, it is observed that increasing the level of zeatin has inhibitory effect on shoot height, the number of leaves, and the number of shoots. According to the effect of zeatin on the number of shoots, this result is converse with Musallam et al. (2011) and Chalak and Elbitar (2006) results. Musallam et al. (2011) reported that increasing zeatin levels (increments of 0.4 mg/L up to 1.6 mg/L) increased the number of shoots from 2.1 to 4.8 and decreased shoot length from 2.44 to 1.78. Moreover, Chalak and Elbitar (2006) showed that increasing zeatin levels increased the number of shoots from and decreased shoot length. Al-Safadi and Elias (2011) study showed that 1.0 and 2.0 mg/L of zeatin had no effect on shoot multiplication; they caused regeneration and callus formation respectively. However, Al-Safadi and Elias (2011) found that MS medium containing 2.0 mg/L zeatin riboside, 0.1 mg/L GA_3 and 1.0 mg/L NAA was the optimum medium for adventitious shoots proliferation from *C. spinosa* stem cutting.

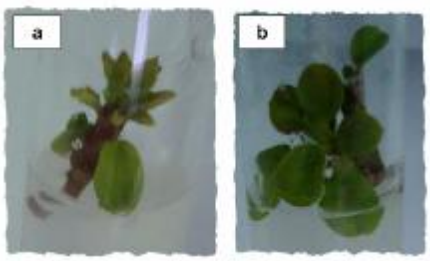

Figure 4.3: Shoots proliferated from *Capparis spinosa* nodal segment on MS medium supplemented with (a) 2.0 mg/L zeatin, (b) 0.5 mg/L zeatin.

4.1.3 Callus Induction and Culture

4.1.3.1 Callus Induction

The effect of light condition on callus induction from leaf segments was studied after five weeks of culture; results indicate that 100% of leaf explants placed on callus induction medium resulted in callus induction in dark condition, however, under light conditions, callus induction percentage dropped to 41%.

After four weeks of culture, the induced callus was weighted by sensitive balance and the callus fresh weights were recorded to evaluate the optimum basal media (MS and B5) for callus induction. The significance between treatments were studied by T-test for comparing means and the results show significant differences ($p < 0.05$) between the two tested media pertaining to their effect on callus induction. MS medium gave higher callus fresh weight than B5 medium. The mean callus fresh weight was 105.03 mg ± 6.54 when using the MS compared to 69.58 mg ± 3.12 with B5 medium (Figure 4.4). The present result is compatible with Anburaj et al. (2011) result on *Cleome viscose* (Capparidaceae) callus induction. They reported that leaf explants cultured on MS medium gave higher callus fresh weight than B5 medium. The mean callus fresh weight was 84.1 mg ± 0.55 when MS was used compared to 37.4 mg ± 0.55 with B5 medium. Accordingly, MS medium is considered an optimal basal media for callus induction in this study.

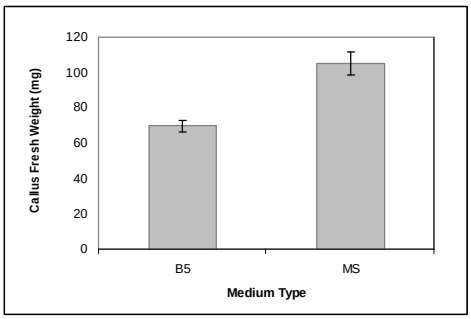

Figure 4.4: The effect of basal media types supplemented with 0.5 mg/L 2,4-D on callus induction from *C. spinosa* leaves after four weeks of culture. Columns represent mean callus fresh weight ± SE (standard error). Sample size (n) = 12.

To evaluate the optimum concentration of 2,4-D in callus induction, the significance between treatments was tested with the analysis of variance by One-Way ANOVA. Results show significant differences ($p < 0.05$) between the three tested media pertaining to their effect on callus induction. MS medium supplemented with 0.5 mg/L 2,4-D induced higher callus fresh weight compared to media where 0.0, or 0.1 mg/L 2,4-D was added. The mean callus fresh weight was 217.19 mg when using MS medium containing 0.5 mg/L 2,4-D compared to 46.08 mg and 28.70 mg when the MS medium supplemented with 0.1 and 0.0 mg/L 2,4-D respectively were used (Figure. 4.5).

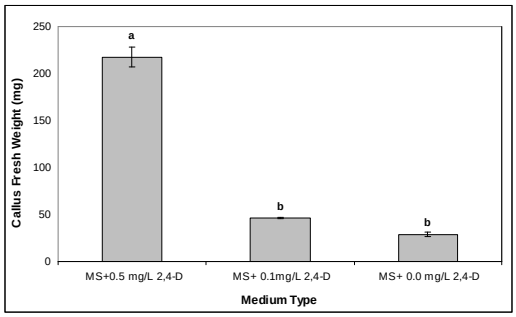

Figure 4.5: The effect of different 2,4-D concentrations on callus induction from *C. spinosa* leaves after four weeks of culture. Columns represent mean of callus fresh weight ± SE (standard error). Different letters accompanying each bar indicate a significant difference by Fisher LSD ($p < 0.05$). Sample size = 12.

The auxin generally used for callus induction is 2,4-D, but NAA and IAA are also used (Afshari et al., 2011). 2,4-D is essential for the induction of callus in many plants at different levels. For example, Medlar a dicotyledonous medicinal plant only needs 0.2 mg/L 2,4-D while ginseng cell culture needs highest concentration of 2,4-D reaches 12 mg/L (Wang et al., 2009). In the current study, it is found that 0.5 mg/L 2,4-D in MS medium is the optimum concentration for callus induction compared to the other concentrations. Wang et al. (2009) showed that the MS medium with 0.5 mg/L 2,4-D and 1.0 mg/L BA was the optimum for callus induction from caper. Moreover, Al-Safadi and Elias (2011) reported that the best growth medium for callus induction from caper leaf was MS medium containing 1.0 mg/L BA and 0.1mg/L NAA and MS medium supplemented with 2.0 mg/L 2,4-D, 1.0 mg/L NAA, and 0.1 mg/L GA_3.

The callus induction growth curve was generated by taking the fresh weight of induced callus from three Petri-dishes every week during five weeks of culture. The growth curve of callus induction is presented in Figure 4.6. According to the shape of the curve (S shape), the best time for callus subculture is the time between the 3rd and 4th week because the curve levels off after that to move to the stationary phase. In the fourth week callus was at the end of lag phase. The growth rate ratio was 4.92.

To the best of my knowledge, it is the first report where a growth curve from callus induction is generated.

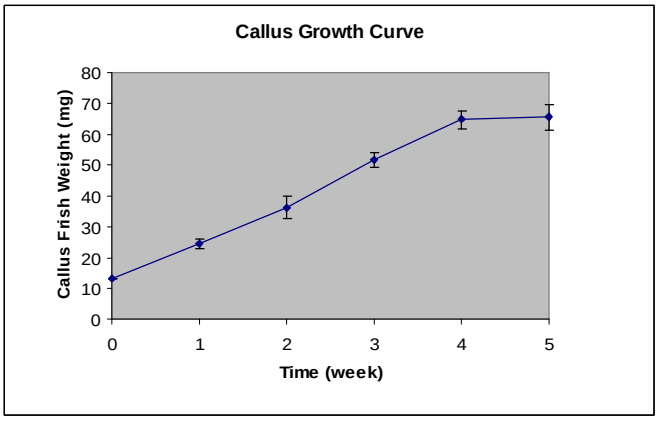

Figure 4.6: Growth curve illustrating change in fresh weight of *C. spinosa* callus induced on full strength MS medium supplemented with 0.5 mg/L 2,4-D in dark condition. Initial weight of inoculum was 13.3 mg (fresh weight). Sample size (n) = 12. The growth rate ratio was 4.92.

4.1.3.2 Callus Growth and Subculture

To find out the optimum medium for callus growth, the fresh weight of subcultured callus in five different types of media was taken after four weeks. The significance between treatments was tested with the analysis of variance by One-Way ANOVA, and the results have shown significant differences ($p < 0.05$) between the five tested media pertaining to their effect on callus growth. MS medium supplemented with 1.0 mg/L 2,4-D and 1.5 mg/L BA was the appropriate medium for callus growth than the four other media. The mean callus fresh weight was 272.27 mg when using MS medium containing 1.0 mg/L 2,4-D and 1.5 mg/L BA compared to the four other media (Figure 4.7). This result agrees with Wang et al. (2009) in which the same growth medium was used for callus growth and similar results were obtained.

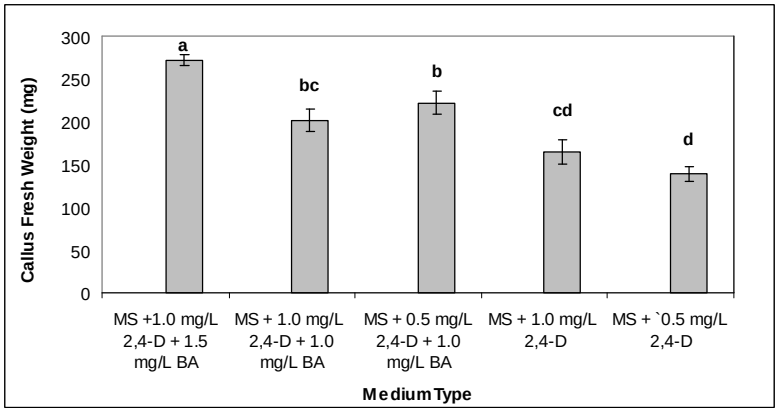

Figure 4.7: The effect of different media types on callus growth after four weeks of culture. Columns represent mean of callus fresh weight ± SE (standard error). Different letters accompanying each bar indicate a significant difference by Fisher LSD ($p < 0.05$). Sample size (n) = 12.

To evaluate the effect of different cytokinin types together with light or dark on callus growth, callus texture, and color, 1.5 mg/L of different PGR types including Zeatin, TDZ, 2ip, and BA were used in full strength MS media and 1.0 mg/L 2,4-D. Data was recorded after four weeks and presented in Table 4.6.

Table 4.6: Average fresh callus weight in mg that were cultured in MS media supplemented with 1.0 mg/L 2,4-D and 1.5 mg/L of different PGR types including Zeatin, TDZ, 2ip, and BA under different light condition after four weeks of subculture. Sample size (n) = 16.

Medium Type	**Light Condition**	**Mean Callus Fresh Weight (mg) ± SE**
MS + 1.0 mg/L 2,4-D + 1.5 mg/L Zeatin	Light	208.59 ± 21.88
MS + 1.0 mg/L 2,4-D + 1.5 mg/L Zeatin	Dark	256.33 ± 19.98
MS + 1.0 mg/L 2,4-D + 1.5 mg/L 2ip	Light	192.21 ± 5.82
MS + 1.0 mg/L 2,4-D + 1.5 mg/L 2ip	Dark	166.91 ± 13.72
MS + 1.0 mg/L 2,4-D +1.5 mg/L TDZ	Light	207.99 ± 17.11
MS + 1.0 mg/L 2,4-D + 1.5 mg/L TDZ	Dark	192.87 ± 16.79
MS + 1.0 mg/L 2,4-D + 1.5 mg/L BA	Light	148.85 ± 20.22
MS + 1.0 mg/L 2,4-D + 1.5 mg/L BA	Dark	285.22 ± 20.34

The effect of cytokinins types together under light or dark conditions on callus growth was tested. Two-way ANOVA showed that cytokinin types, light conditions, and the interaction between cytokinin types and light conditions gave significant effect on callus growth ($p < 0.05$). MS medium supplemented with 1.0 mg/L 2,4-D and 1.5 mg/L BA that was cultured under dark condition gave the highest mean callus fresh weight 285.22 mg compared to the other media (Figure 4.8). This result is in agreement with Afshari et al. (2011) result which showed that the use of 2,4-D was very effective on *Brassica napus* callusing, especially when combined with cytokinin (BA). On the other hand, Afshari et al. (2011) reported that light had a significant effect on increasing *Brassica napus* callus fresh weight compared to callus cultured under dark condition.

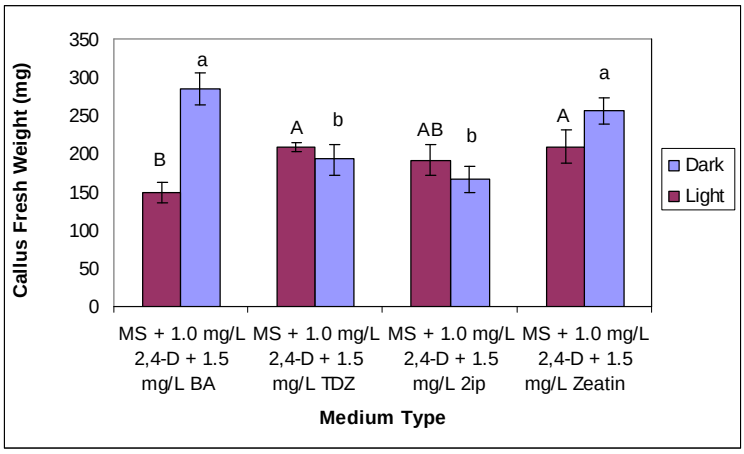

Figure 4.8: The effect of light condition together with different cytokinins types on callus growth after four weeks of culture. Columns represent mean of fresh callus weight ± SE (standard error). The blue columns represent the dark condition whereas the pink columns represent the light condition. Different letters accompanying each bar indicate a significant difference by Fisher LSD ($p < 0.05$). Capital letters were used to show light condition and small letters for dark condition. Sample size (n) = 16.

According to callus texture and color, the callus that has been cultured on MS medium supplemented with 1.0 mg/L 2,4-D and 1.5 mg/L TDZ under light condition was compact and gave different colors (violet, yellow, and green) compared to the one that was cultured under dark which was compact in texture and yellow in color. Callus which was grown on medium containing BA under light condition was compact and appeared in different colors like violet, green, and yellow. However, when callus was grown under dark, it was friable and had a yellow color (Table 4.7) and (Figure 4.9).

Table 4.7: Callus color and texture that were observed when callus were cultured in MS media supplemented with 1.0 mg/L 2,4-D and 1.5 mg/L of different PGR types including Zeatin, TDZ, 2ip, and BA under different light condition after four weeks of subculture.

Cytokinins Types	Light Condition	Callus Color	Callus Texture
Zeatin	Light	Different colors; yellow, gray, and green	Compact
Zeatin	Dark	Different colors; yellow and pale green	Compact
2ip	Light	Yellow	Compact
2ip	Dark	Yellow	Compact
TDZ	Light	Different colors; yellow, violet, and green	Compact
TDZ	Dark	Different colors; yellow and gray	Compact
BA	Light	Different colors; violet, green, and yellow	Compact
BA	Dark	Yellow	Friable

This study demonstrated that the type of cytokinins and light condition have significant effect on callus color and texture. Afshari et al. (2011) studied the effect of light condition on callus color and they showed that newly initiated callus from cotyledon was creamy in the dark and mostly green in the light. Also, they investigated the cytokinins effect on callus color; they reported that the presence of BA in callus media produced pale green color and this can be explained by the presence of BA, a type of cytokinins and cytokinins tend to promote chlorophyll formation. Moreover, Daffalla et al. (2011) reported that the auxin 2,4-D and the cytokinin TDZ appear to induce callus but in different forms. When 2,4-D used, embryos of *Boscia senegalensis* (Capparidaceae) produced a pale yellow colored friable callus, whereas, when embryo explants cultured on TDZ supplemented medium, scattered protuberances were formed on their surfaces.

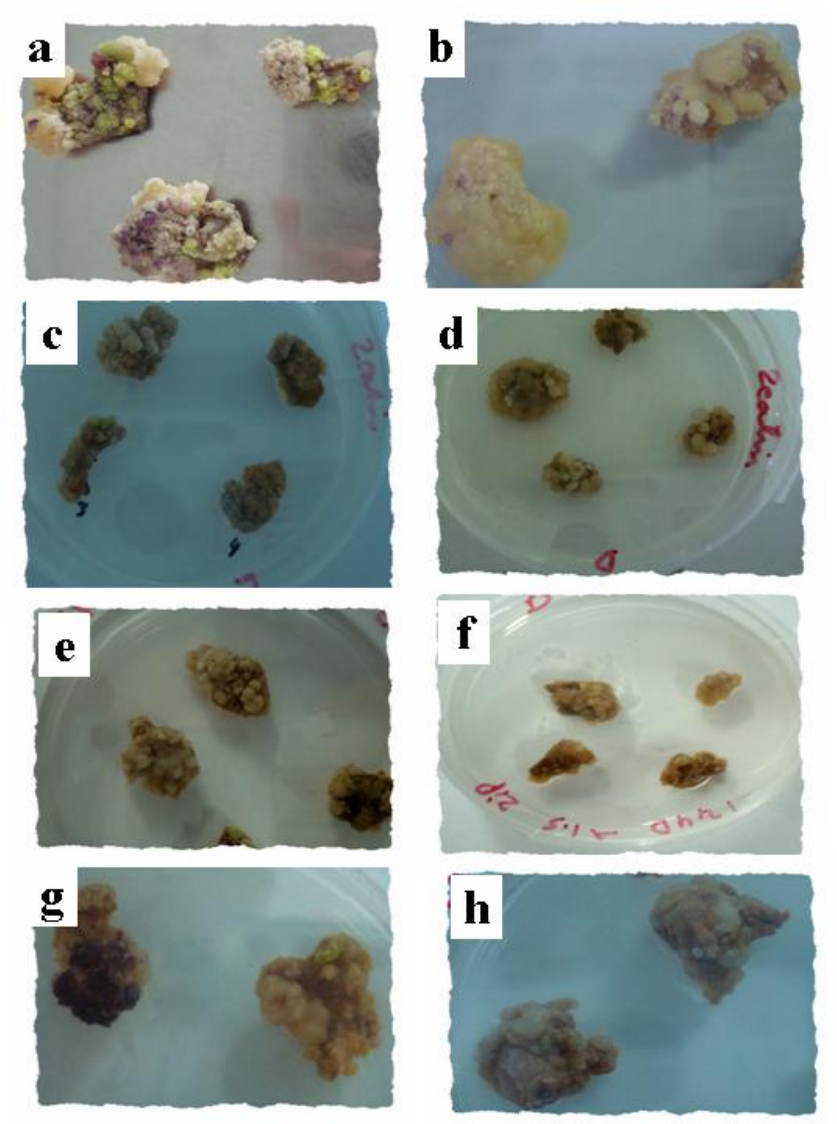

Figure 4.9: Callus growth after four weeks of culture on full strength media containing 1.0 mg/L 2,4-D and 1.5 mg/L of different types of cytokinins under different light conditions. (a) BA under light condition, (b) BA under dark condition, (c) zeatin under light condition, (b) zeatin under dark condition, (e) 2ip under light condition, (f) 2ip under dark condition, (g) TDZ under light condition; (h) TDZ under dark condition.

4.1.4 *In Vitro* Root Culture

4.1.4.1 *De Novo* Root Induction from Leaf Disc

The effect of auxin type on root induction from *C. spinosa* leaf discs was evaluated after six weeks of culture. Under light condition, no root induction was observed in all treatments. Under dark condition, adventitious roots were induced in different media types at different rates. The root induction response, roots number and length are presented in Table 4.8. In the present study the direct root induction from *Capparis spinosa* leaf discs was observed. Many previous studies on other plant species showed that leaf segments were the best explants for root induction. In Pandey et al. (2010)

study, root growth from the leaf segment of *Rauwolfia serpentine* on MS medium containing 4.5 mg/L NAA was obtained. Moreover, Khalafalla et al. (2009) were able to derive roots from the leaf of *Vernonia amygdalina* Direct root growth from the leaf and hypocotyls explants of *Cichorium intybus* L. had also been reported by Nandagopal and Ranjitha Kumari (2007). Furthermore, root induction had been obtained from the leaf of *Orthosiphon stamineus* cultured on MS medium supplemented with 3.0 mg/L of IAA in Kiong Ling et al. (2009) study. Kiong Ling et al. (2009) justified that the leaf segments were good starting tissue for root induction may due to the presence of cells associated with the leaf veins (vascular tissue), which can be readily stimulated by adding auxins and phytohormones can easily be manipulated to direct pluripotent cells to a particular cell fate.

In the present study, absence of lighting has important role in root induction and the complete dark culture was more effective for the production of roots than light. This fits with the natural system where roots grow in soil far from sun light. The present result was agreed to the Nandagopal and Ranjitha Kumari (2007) root culture which produced more and faster adventitious roots in dark condition than light condition. Pierik (1987) also mentioned that light commonly has a negative effect on root formation. Moreover, Afshari et al. (2011) reported that root growth can be improved in dark induction.

No root induction was observed in auxins deprived medium. The vital role of auxins on rooting has also been previously reported by Khalafalla et al. (2009) and Kiong Ling et al. (2009). The MS medium supplemented with 1.0 mg/L NAA gave the highest root induction response (62.5%), followed by 0.1 mg/L NAA and 0.5 mg/L NAA (56.25% and 25%) respectively. On the other hand, IBA at different concentrations gave lower root induction response with less than 19%. No effect of IAA at the concentrations used on root induction was observed. According to the number of roots and root length, the statistical significance between treatments was analyzed by Kruskal-Wallis ANOVA, the results have shown a statistically significant differences ($p < 0.05$) between the 10 tested media. The MS medium supplemented with 1.0 mg/L NAA gave the highest number of roots per culture and the longest root per culture compared with the other auxin types and concentrations.

Table 4.8: Rooting percentage, average number of roots, and root length of *in vitro* root induction from leaves of *C. spinosa* in dark condition in response to different treatments of auxins after six weeks of culture. Sample size (n) = 16.

Type and Concentration of Auxins (mg/L)	**Percentage of Response (%)**	**Mean of Root Number / Culture (Mean ± SE)**	**Mean of Root Length / Culture (cm) (Mean ± SE)**
NAA (0.1)	56.25	1.19 ± 0.42	0.56 ± 0.18
NAA (0.5)	25.00	0.94 ± 0.49	0.33 ± 0.16
NAA (1.0)	62.50	1.69 ± 0.50	0.57 ± 0.16
IBA (0.1)	18.75	0.25 ± 0.14	0.14 ± 0.09
IBA (0.5)	0	0	0
IBA (1.0)	12.50	0.19 ± 0.14	0.12 ± 0.08
IAA (0.1)	0	0	0
IAA (0.5)	0	0	0
IAA (1.0)	0	0	0`
Control	0	0	0

This result supports previous report by Pandey et al. (2010) when showed an evidence of root induction on MS medium containing 4.5 mg/L NAA. Khalafalla et al. (2009) showed that IBA was more effective than NAA in *V. amygdalina* adventitious root induction. Moreover, Kiong Ling et al. (2009) reported that IAA at 3.0 mg/L was the best auxins in *O. stamineus* for adventitious roots formation. The reasons of lower rooting efficiency from IBA and no rooting response in IAA treatments in caper plant might be due to some factors like genetic variation between species, in which different species responded to same type of plant growth regulator differently and some plants species were reported to response better in IBA or IAA treatments (Pandey et al., 2010; Khalafalla et al., 2009; Kiong Ling et al., 2009; Nandagopal and Ranjitha Kumari, 2007). Also, Pierik (1987) showed that the weak auxin IAA is often used during rooting of herbaceous plants but woody plants responds better with IBA and NAA.

In another experiment, the effect of combination of auxins (NAA with 2,4-D, IBA, or IAA) added to MS medium on root induction was studied. Root and callus induction percentage are presented in Table 4.9.

Table 4.9: Percentage of root induction and callus induction from leaves of *C. spinosa*, in dark in response to different auxins combinations after six weeks of the culture. Sample size (n) = 16.

Types and Concentrations of Auxins (mg/L)	**Percentage of Root Induction (%)**	**Percentage of Callus Induction (%)**
NAA (1.0) + 2,4-D (0.1)	0	24
NAA (1.0) + 2,4-D (0.5)	0	37
NAA (1.0) + 2,4-D (1.0)	0	66.6
NAA (1.0) + 2,4-D (1.5)	0	83.6
NAA (2.0) + 2,4-D (1.0)	0	100
NAA (2.0) + 2,4-D (1.5)	0	91.5
NAA (1.0) + IBA (0.1)	0	0
NAA (1.0) + IBA (0.5)	4.1	0
NAA (1.0) + IBA (1.0)	12.5	0
NAA (1.0) + IBA (1.5)	6.25	0
NAA (2.0) + IBA (1.0)	2	0
NAA (2.0) + IBA (1.5)	12.5	0
NAA (1.0) + IAA (0.1)	0	0
NAA (1.0) + IAA (0.5)	0	0

It is observed that media with 1.0 mg/L NAA plus IAA at 0.1 or 0.5 mg/L resulted in absence of root induction or callusing. Supplementing the media with 1.0 or 2.0 mg/L NAA with different 2,4-D levels resulted in callus induction from leaf segments. Root induction was effectively observed when the two auxins IBA and NAA were used, with highest induction percentage (12.5%) at 1.0 mg/L NAA with 1.0 mg/L IBA and 2.0 mg/L NAA with 1.5 mg/L IBA. Contrary to our result, in Nandagopal and Ranjitha Kumari (2007) study, the root induction was higher in a combination of low concentration of NAA with high concentration of IAA or IBA. The combination of IBA and NAA in Pandey et al. (2010) study showed good response in terms of root formation, however, IBA at 2.0 mg/L in combination with 4.5 and 5.0 mg/L of NAA produced 28 and 30 roots respectively, while the combination of 0.5 mg/L BAP and 2.0 mg/L NAA produced only callus. In the current study, when NAA is combined with 2,4-D no root induction was observed and callus induction was noted in different percentages (Table 4.9). This result is in agreement with Afshari et al. (2011) study. When NAA is combined with 2,4-D, no rooting or rhizogenesis were observed in *Brassica napus* cultures. Using higher concentration of 2,4-D (2.0 mg/L) resulted in low rhizogenesis and root formation.

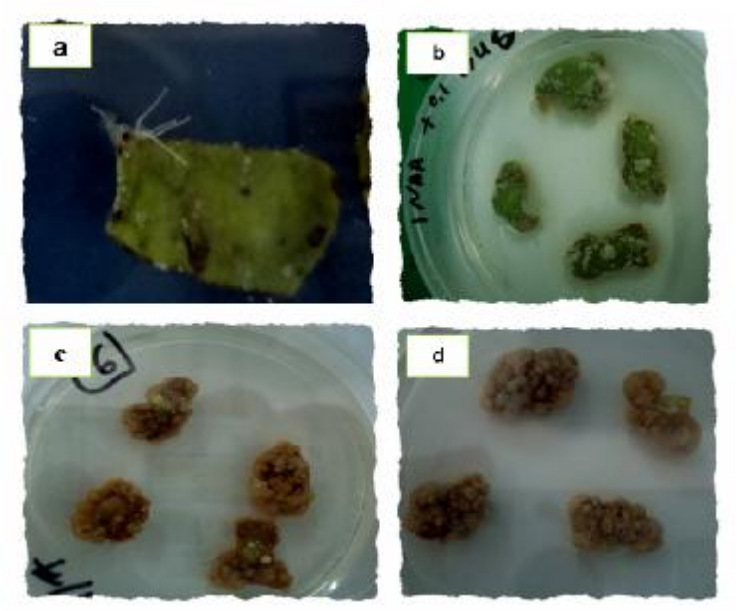

Figure 4.10: The effect of different combination of auxins on root or callus induction. (a) Roots induced on MS medium containing 1.0 mg/L NAA and 0.5 mg/L IBA, (b) Callus induced on MS medium containing 1.0 mg/L NAA and 0.1 mg/L 2,4-D, (c) Callus induced on MS medium containing 2.0 mg/L NAA and 1.5 mg/L 2,4-D, (d) Callus induced on MS medium containing 2.0 mg/L NAA and 1.0 mg/L 2,4-D.

Sugar type and concentration has a significant effect on root induction from leaves of *C. spinosa* (Table 4.10). In this study, no root formation was observed in sugar deprived media and this demonstrates the necessity of the carbon source in adventitious roots induction. MS medium supplemented with 1.5 mg/L NAA and 30 g/L sucrose gave 41.6% rooting percentage, while media contained 30 g/L fructose or 15 g/L fructose produced 25% and 8.3% rooting percentage respectively. According to the number of roots and roots length, the significance between treatments was analyzed by Kruskal-Wallis. The results showed statistically significant differences ($p < 0.05$) between the tested media. MS medium containing 1.5 mg/L NAA supplemented with 30 g/L sucrose gave the highest number of roots and root length per culture compared with other carbon sources and concentrations. This result is agreed to the Hossain et al. (2005) results which showed that medium containing 0.2 mg/L NAA and sucrose (30 g/L) produced 70% root induction and highest root length of *Centella asiatica* L. rather than other carbon sources like glucose and maltose. Differing to the present result, Al-Khateeb (2002) found that the type of sugar had no significant effect on rooting percentage and root number of *Phoenix dactylifera* L. while types and concentrations of sugars significantly affected root length. It is well

documented that species vary in growth response if carbon source is modified (Pierik, 1987).

Table 4.10: Percentage of root induction, average number of roots, and root length of *in vitro* root induction from leaves of *C. spinosa* in dark condition in response to different sugar types and levels used in MS media supplemented with 1.5 mg/L NAA after six weeks of culture. Sample size (n) = 12.

Carbon Source Types and Concentrations	Percentage of Root Induction (%)	Mean of Root Number / Culture (Mean ± SE)	Mean Root Length / Culture (cm) (Mean ± SE)
Sugar Free	0	0	0
Sucrose (30g/L)	41.6	0.67 ± 0.26	0.13 ± 0.05
Sucrose (15g/L)	0	0	0
Fructose (30g/L)	25	0.42 ± 0.23	0.09 ± 0.05
Fructose (15g/L)	8.3	0.17 ± 0.17	0.03 ± 0.03
Sorbitol (30g/L)	0	0	0
Sorbitol (15g/L)	0	0	0

According to the effect of explant source on root induction, leaf discs that were taken from the *in vitro* growing plants gave higher root induction percentage (68.8%) than *ex vitro* growing plants (43.8%). Also, the roots that were induced from *in vitro* leaves were longer and appeared as a fibrous root mass (Figure 4.11). This result can be explained by the fact that the root formation is more easily induced in juvenile than old plant parts (Pierik, 1987). Additionally, the surface sterilization of*ex vitro* leaves is negatively affects leaf structure and consequently it altered the root induction.

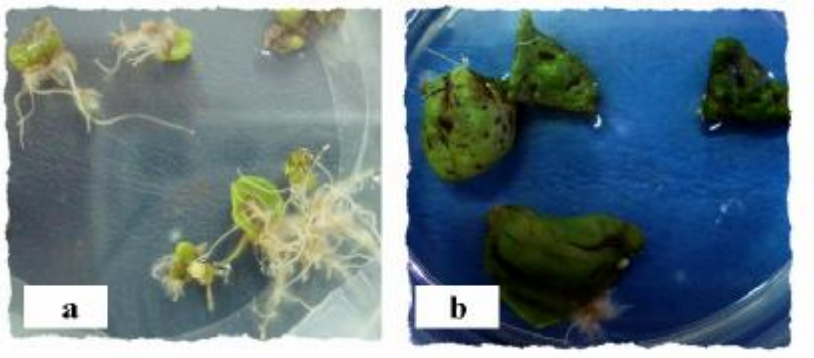

Figure 4.11: Root induction on MS medium supplemented with 1.0 mg/L NAA after six weeks of culture from, (a) *in vitro* leaves, (b) *ex vitro* leaves.

4.1.4.2 *In Vitro* Root Culture and Growth

4.1.4.2.1 Root Growth in Semi-solid Media

The root growth was observed after six weeks of culturing leaf segments on different types of semi-solid media that supplemented with different levels and types of auxins. The root growth was determined by measuring the roots growth area in cm^2 (Table 4.11).

Table 4.11: Average root growth area (cm^2) of *in vitro* root culture from leaves of *C. spinosa* in dark condition in response to different treatments of auxins in semi-solid media after six weeks of culture. Sample size (n) =12.

Medium Type	**Mean Root Growth Area (cm^2) ± SE**
MS Free	0.38 ± 0.07
MS + 1.0 mg/L NAA	1.73 ± 0.60
MS + 2.0 mg/L NAA	2.42 ± 0.71
MS + 1.0 mg/L NAA + 1.0 mg/L IBA	0.56 ± 0.23
MS + 2.0 mg/L NAA + 1.0 mg/L IBA	2.40 ± 0.84

According to root growth area, the significance between treatments was analyzed by Kruskal-Wallis ANOVA, the results have shown statistically significant differences ($p < 0.05$) between the five tested media. MS medium supplemented with 2.0 mg/L NAA and IBA at 0.0 or 1.0 mg/L produced the largest root growth area (2.42 cm^2 and 2.40 cm^2 respectively) compared to the other tested media.

On the other hand, the effect of leaf segments from two different sources, potted and *in vitro* grown plants, and different NAA levels (1.0 and 2.0 mg/L) on root growth was tested. The areas of root growth in cm^2 are presented in Table 4.12.

Table 4.12: Average root growth area (cm^2) of *in vitro* root culture from leaves of *C. spinosa* in dark and in response to different NAA levels in MS media and different explants source after six weeks of culture.

Medium Type and Leaf Segments Source	**Mean Root Growth Area (cm^2) ± SE**
1.0 mg/L NAA (*In vitro*)	1.69 ± 0.48
2.0 mg/L NAA (*In vitro*)	1.77 ± 0.59
1.0 mg/L NAA (*Ex vitro*)	0.63 ± 0.16
2.0 mg/L NAA (*Ex vitro*)	0.60 ± 0.13

According to root growth area, the significance between treatments was analyzed by Kruskal-Wallis ANOVA, the results have shown no statistically significant differences ($p > 0.05$) between the tested media. Root growth from *in vitro* leaves on MS medium containing 2.0 mg/L NAA gave the highest growth area (1.77 cm^2 ± 0.59) followed by *in vitro* leaves which were cultured on MS medium supplemented with 1.0 mg/L NAA (1.69 cm^2 ± 0.48).

4.1.4.2.2 Root Growth in Liquid Media

The effect of liquid media with NAA at 1.0 or 2.0 mg/L on root growth was tested by measuring the growth area of roots every two weeks of culture. The mean growth areas in cm^2 in two weeks interval during a period of eight weeks are presented in Table 4.13. Result did not show significant differences between the two tested media pertaining to their effect on root growth ($p > 0.05$). The MS liquid medium supplemented with 1.0 mg/L NAA gave higher growth than MS liquid medium containing 2.0 mg/L NAA where the mean root growth area was 4.08 ± 1.13 cm^2 when using 1.0 mg/L NAA compared to 3.00 ± 0.43 cm^2 when the 2.0 mg/L NAA was added to the medium.

Table 4.13: The effect of NAA concentration on root growth from caper leaves in liquid media during eight weeks of culture. The final column represented the root growth area increased from the time of culture starting to the six weeks of culture. Sample size = 6.

Medium Type	**Mean Root Growth Area (cm^2) ± SE**					
	0 week	**2 weeks**	**4 weeks**	**6 weeks**	**8 weeks**	**Net Area Growth (0-6 weeks)**
1.0 mg/L NAA	4.67 ± 1.43	6.50 ± 1.95	7.96 ± 2.07	8.75 ± 2.12	7.96 ± 2.07	4.08 ± 1.13
2.0 mg/L NAA	1.71 ± 0.32	3.75 ± 0.51	4.04 ± 0.48	4.71 ± 0.52	4.54 ± 0.72	3.00 ± 0.43

The data for *C. spinosa* root growth area are presented in (Figure 4.12). A root tissue (4.67 cm^2 ± 1.43) cultured in 8.0 ml full-strength MS liquid medium supplemented with 1.0 mg/L NAA and under continuous agitation (80 rpm) showed most significant root growth increase during the first four weeks of culture. The maximum growth area of roots (8.75cm^2 ± 2.12) was obtained at the 6^{th} week of culture and later at the 6^{th} week the growth was slowly declined (7.96 cm^2± 2.07) and roots began to appear

brown in color (Figure 4.12 and 4.14). It is concluded that the best time for root subculture is the time between the 4th and 6th week because the curve attend to move to the stationary phase.

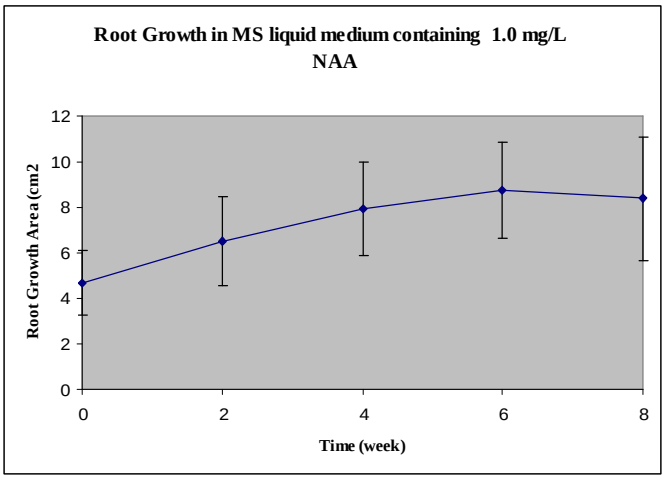

Figure 4.12: Time course of *Capparis spinosa* root growth in full strength MS medium supplemented with1.0 mg/L NAA. Initial root growth area of inoculum was 4.67 cm^2 in 8.0 ml medium.

In full-strength MS liquid medium supplemented with 2.0 mg/L NAA, the most significant root growth expansion was during the first two weeks of culture (Figure 4.13). The maximum growth area of roots (4.71 cm^2 ± 0.52) was obtained at the 6th week of culture and later at the 6th week the growth was gradually decreased (4.54 cm^2± 0.72) and roots began to appear brown in color.

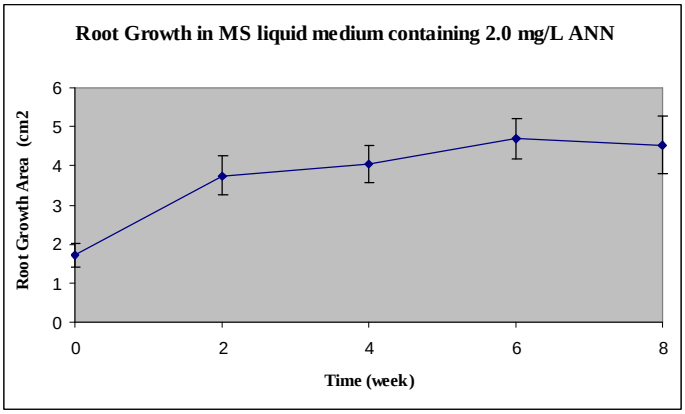

Figure 4.13: Time course of *Capparis spinosa* root growth in full strength MS medium supplemented with 2.0 mg/L NAA. Initial root growth area of inoculum was 1.71 cm^2 in 8.0 ml medium.

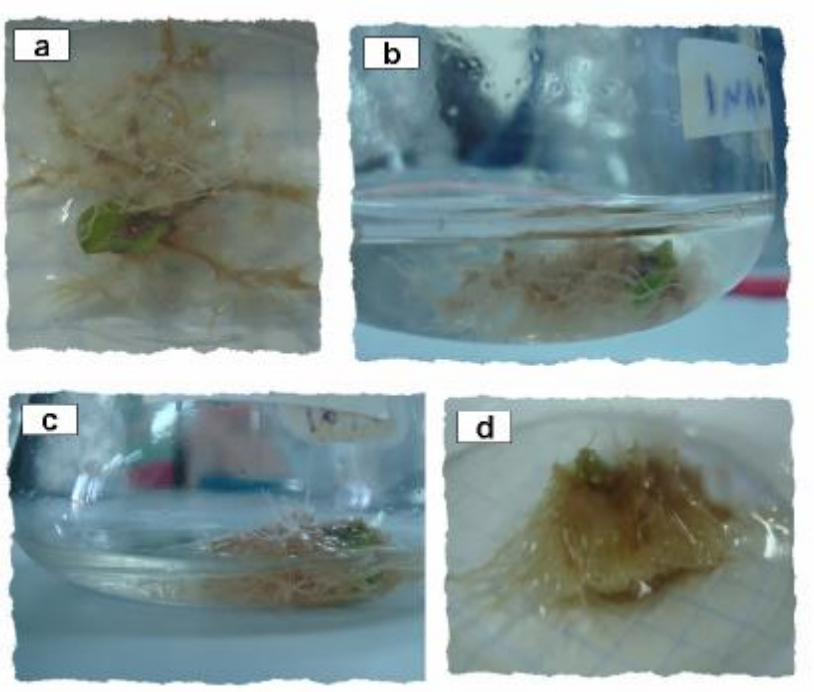

Figure 4.14: Root growth in MS liquid media supplemented with 1.0 mg/L NAA. (a) at starting day of culture, (b) after two weeks of culture, (c) after six weeks of culture, (d) after eight weeks of culture.

In summary, root growth in liquid media or semi-solid media is better than in solid media. Pierik (1987) reported that the formation of adventitious roots especially in woody plants is generally poor on solid media and liquid medium is sometimes preferred in woody plants. In the present study, the root growth was measured by calculating the root growth area during growing period and the growth area was taken every two weeks until the eighth week of culture. This method was used instead of taking root fresh weight to avoid possible contamination during weighing and losing cultures.

In the two media used, the most significant in increase root growth was observed during the first two weeks of culture. The maximum growth area of roots was measured at the 6^{th} week of culture, and later on at the growth was slowly declined and roots began to browning. The decline of root growth might be due to the accumulated inhibitors and nutrients decline. Accordingly, it is necessary to change the growth media and do subculture every six weeks of root culture. Similarly, in Khalafalla et al. (2009) study, the *Vernonia amygdalina* root biomass was increased after five weeks of culture and the root culture was maintained up to six weeks. Also, Nandagopal and Ranjitha Kumari (2007) reported that the biomass of root culture of *Cichorium intybus* was increased after six weeks of culture and the root culture was maintained up to the eight weeks.

To the best of my knowledge, no previous studies were conducted to put a protocol for the *in vitro* root induction and culture of *C. spinosa.* The present study is carried out based on the fact that although all parts of caper are pharmaceutically useful, roots are the principle material used in our traditional medicine to treat many diseases like rheumatism and back and joint pains (Hamed et al., 2007). In the present study, a reproducible method for high frequency root induction and growth from leaf segments without threatening the wild life and natural resources was established.

4.2 Extraction of Secondary Metabolites

4.2.1 Determination of TSS in Vinegar

Following to the evaporation of liquid phase in apple and grape vinegar, 1.0 ml of each gave 0.0409 g and 0.0947 g TSS respectively (appendix table 17).

4.2.2 Extraction Yields

The extraction yields for each plant material in different solvent systems were recorded. Extraction yields in this study ranged between 89.34% and 2.06 %. According to the results, leaves extract in apple vinegar has the highest extraction yield with 89.34%. Apple vinegar extracts in general gave the highest extraction yield compared to other solvents (Table 4.14).

Table 4.14: Extraction yields of plant materials that were extracted with different solvents.

Extracted Plant Material	Extraction Yield (%)		
	Grape Vinegar	Apple Vinegar	Ethyl Alcohol
Root bark	29.28	45.03	2.27
Leaves	48.86	89.34	25.01
Dry callus	32.78	64.47	24.37
Fresh callus	2.06	43.16	Not extracted

The dry callus was obtained by drying fresh callus on desiccators for five days, fresh and dry callus were weighted and the water amount in callus was calculated. Fresh callus weight was 10.56 g and dry callus weight was 1.18 g. The weight of water in callus was 9.38 g.

4.2.3 Evaluation of Vinegar Solubility

In order to test the dissolving ability of vinegar, the precipitate from dried callus obtained from vinegar extract was re-extracted with different solvents (chloroform, distilled water, 95% ethyl alcohol) and the extraction yield of each one was calculated (Table 4.15).

Table 4.15: The extraction yields of the precipitate of dried callus extract in apple vinegar which were further extracted with different solvents.

Solvent Type	Starting Weight (g)	Final Weight (g)	Yield (%)
Chloroform	2.5868	0.0152	0.59
Distilled water	2.4690	0.0135	0.55
Ethyl alcohol (95%)	2.3904	0.0115	0.48

The results indicate the dissolving power of apple vinegar to dry callus, <1% yield was isolated when solvents with different polarity were used to re-extract the precipitate. This result justifies the further evaluation of natural liquids as extracting solvents for natural product.

4.2.4 Spectrophotometric Quantification of Vinegar Extracts

The absorbance of root-bark apple vinegar extract (44 mg/ml) was measured and compared with the absorbance of pure apple vinegar. Results show an increase in the absorbance in extracted sample compared to pure vinegar (Figure 4.15). Similarly, the absorption of root bark extracts in grape vinegar increased in comparison to pure grape vinegar (Figure 4.16). Spectrophotometry is reliable tool that could be used to follow up and optimize extraction time.

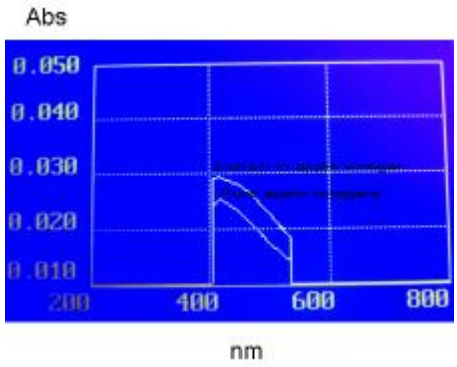

Figure 4.15: Absorbance comparison between pure apple vinegar (lower line) and root bark extract (upper line) in apple vinegar.

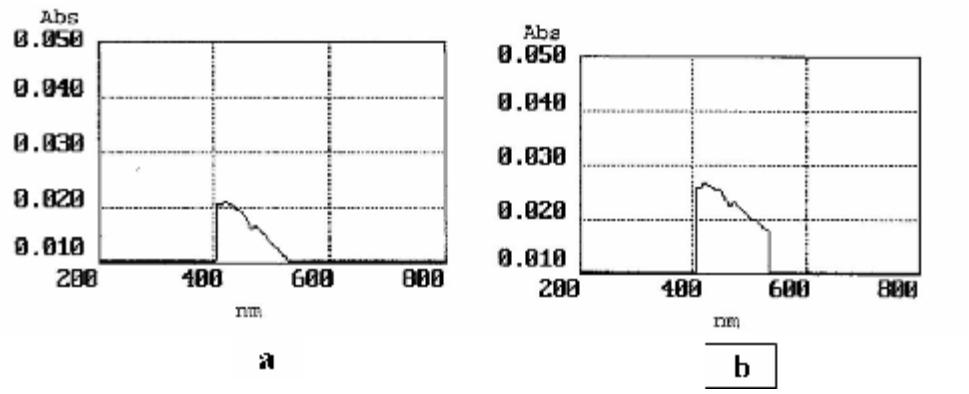

Figure 4.16: Comparison of the magnitude absorbance of (a) pure grape vinegar, (b) root bark extract in grape vinegar.

4.3 Antimicrobial Activities of *C. spinosa* Extracts

In order to study the antimicrobial activities of caper extracts, the disc diffusion assays was performed (Mahasneh, 2002; Mahasneh et al. 1996). Different types of plant materials (root bark, leaves, and callus) were extracted by different solvents (ethanol, apple and grape vinegar), were used in this assay. Different concentrations of plant extracts (4000, 2000, 1000, 500 µg/disk) were used to test their antimicrobial activity. The results have shown that all tested caper extracts at different concentrations did not show any activities against gram negative "*Escherichia coli*" (Figure 4.17), gram positive "methicillin-resistant*S. aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA)" (Figure 4.18 and 4.19) bacteria, and the two candida species; *C. albicans* (Figure 4.20) and *C. parapsilosis* (Figure 4.21). The negative controls; DMSO, water, apple and grape vinegars also did not show any antimicrobial activities. On the other hand, positive controls; Ampicillin, Gentamicine, and Sulphamethoxazole/ Trimethoprim resulted in inhibition zone and showed antimicrobial activities.

In this study, no antibacterial activity of caper extracts against the tested*E. coli* strain (001RH) was observed (Figure 4.17).

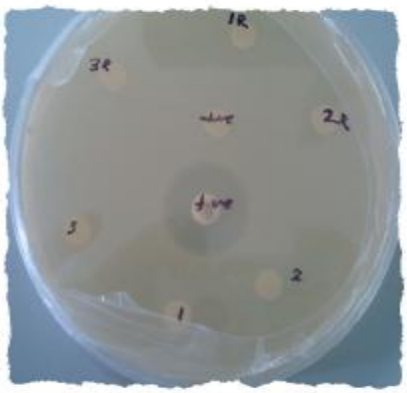

Figure 4.17: Disk diffusion assay of apple vinegar extract of root bark on *e-coli,* different extract concentrations (4000, 2000, and 1000 µg/disk), negative controls (apple vinegar and DMSO), and positive control (Ampacillin) were used. The inhibition zone was observed only around Ampacillin and no inhibition zone around the extracts and negative controls was observed.

Moreover, no antibacterial activity of *C. spinosa* root bark, leaves, and callus extracts at different concentrations against *S. aureus* MRSA and MSSA was observed.

Figure 4.18: Disk diffusion assay of ethanol extracts on *S. aureus* (MRSA), the inhibition zone was observed only around positive controls (Gentamicine and Sulphamethoxazole/ Trimethoprim). No antibacterial activity of leaves, root bark, and callus extracts at different concentrations (4000, 2000, 1000, 500 µg/disk) was observed.

Figure 4.19: Disk diffusion assay of grape vinegar extracts on *S. aureus* (MSSA), the inhibition zone was observed only around positive controls (Gentamicine and Sulphamethoxazole/ Trimethoprim). No antibacterial activity of leaves, root bark, and callus extracts at different concentrations (4000, 2000, 1000, 500 µg/disk) was observed.

According to antifungal activity of *C. spinosa* extracts, the results showed no antifungal activities was observed against the two tested candida species;*C. albicans* (Figure 4.20) and *C. parapsilosis* (Figure 4.21).

Figure 4.20: Disk diffusion assay of apple vinegar extract of root bark on *C. albicans*, no inhibition zone was observed around different extract concentrations (4000, 2000, 1000, 500 µg/disk).

Figure 4.21: Disk diffusion assay of apple vinegar extract of callus on *C. parapsilosis*, no inhibition zone was observed around different extract concentrations (4000, 2000, 1000, 500 µg/disk).

The results in the current study agreed with Darwish and Aburjai (2011) results which showed that the clinical *C. albicans* isolate was found to be resistant to *Capparis spinosa* methanolic root extracts whereas, this extract had antifungal activity against *C. albicans* reference strain with MIC value of 12.5 mg/ml. Also, Boga et al. (2011) reported that the decoction of *C. spinosa* root bark did not show any antibacterial activity against *E. coli* while it had an interesting activity against *Deinococcus radiophilus;* when the decoction was added to the culture medium, the growth rate of *D. Radiophilus* populations decreased significantly,and this result was consistent with the present result. The results of Bouriche et al. (2011) were inconsistent with our results; they reported that the methanolic extract of *C. spinosa* buds showed antimicrobial activities against many organisms like *E. coli, Pseudomonas aeroginosa, Pseudomonas aeroginosa, Staphylococcus aureus,* and *Bacillus subtilis.* However, it did not show antimicrobial activity against *Enterobacter cloacea, Klebsiella pneumoniea, Salmonella typhi*,and *Bacillus megaterium.*Mahasneh (2002) and Mahasneh et al. (1996) results showed that the antimicrobial effect of*C. spinosa* extracts depends on solvent type. Mahasneh (2002) reported that the aqueous crude extract did not show any obvious activity against gram negative bacteria, but the ethanol crude extract exhibit moderate antibacterial and antifungal activity. The butanol extracts at 2000 µg/disc had a very good antibacterial activity against both gram positive and negative bacteria as well as moderate to high antifungal activity against *C. albicans* and *A. flavus.* Moreover, Mahasneh et al. (1996) showed that the petroleum ether and aqueous crude extracts of aerial parts of caper exhibited weak antimicrobial activities against *C. albicans, A. flavus, E. coli, S. typhimurium, S.*

aureus, and B. cerrus. The methanol, hexane, and butanol crude extracts exhibited moderate antibacterial activities against *B. cerrus* and weak antimicrobial activities against *C. albicans, A. flavus, E. coli, S. typhimurium,* and *S. aureus.*

In the present study, *C. spinosa* extracts have not shown any antimicrobial activities against tested organism (Figures 4.17, 4.18, 4.19, 4.20, and 4.21). This result might be explained by different explanations;

1. Isolate types; the clinical isolates were used in this study rather than the standard isolates. The clinical isolates (from Caritas Baby Hospital) might produce different resistance against antimicrobial agents.
2. The extraction procedure; primitive methods of extraction were used in this study, so these procedures might degrade, inhibit, or unable to extract the phytochemicals that have antimicrobial activities.
3. Time of harvesting of plant materials (root bark and leaves) that were used in the test; the time of harvesting might be not appropriate and the phytochemicals that have antimicrobial activities did not reach their optimum concentrations at harvesting time.

CHAPTER 5

Conclusion

In this study, an alternative propagation protocol for caper propagation via plant tissue culture was investigated. Furthermore, plant tissues that have medicinal activities were produced in a large scale. In the present study, the newly harvested seeds of *C. spinosa* are not considered a good starting material for the *in vitro* propagation due to low percentage of germination and strong seed dormancy. Additionally, no effect on seed germination was observed either by changing media types and constituents or by placing seeds under dark or light conditions. Moreover, the age of seeds have a significant effect on germination; two years stored seeds gave high germination percentage compared to newly harvest or one year stored seeds. For shoot proliferation and regeneration from nodal segments, the MS medium containing 0.5 mg/L zeatin was the appropriate medium since it gave the highest number of leaves, shoot height and number of shoots. Moreover, BA at 0.5 mg/L added to the MS medium supplemented with 0.5 mg/L IAA, and 1.0 mg/L GA_3 was the optimum concentration for plant proliferation, since it gave the highest number of leaves compared to the other BA concentrations. In the current study, callus was successfully induced from leaf segments on MS media supplemented with 0.5 mg/L 2,4-D under dark condition. Additionally, there is a significant interaction between cytokinin types and light condition; the MS medium supplemented with 1.0 mg/L 2,4-D and 1.5 mg/L BA and was cultured under dark condition gave the highest callus fresh weight compared to other cytokinins (Zeatin, TDZ, or 2ip). Adventitious roots were successfully induced from leaves of*C. spinosa* on MS medium supplemented with 1.0 mg/L NAA under dark condition. The leaf discs from *in vitro* growing plants gave better adventitious roots compared to leaves from *ex vitro* growing plants. Moreover, the types and concentrations of carbon source have a significant effect on root induction from leaves of *C. spinosa*; MS medium containing 1.5 mg/L NAA and supplemented with 30 g/L sucrose gave the highest number of roots and root length per culture compared to other types and concentrations of carbon sources like fructose and sorbitol. The MS Simi-solid medium containing 2.0 mg/L medium produced the

highest root growth area. In this study, MS liquid medium supplemented with 1.0 mg/L NAA was the optimum medium for root growth. The maximum growth area of roots was obtained at the 6th week of culture and later at the 6th week the growth was slowly declined. For antimicrobial activity of *Capparis spinosa* extracts, results under the experiments settings revealed that all the tested extracts did not show antimicrobial activities against *E. coli*, *S. aureus*, *C. albicans*, and *C. parapsilosis*.

Future Works

Based to the tissue culture part, more studies must be carried out on *in vitro* root culture such as genetic transformation by *Agrobacterium* in order to produce needed and valuable secondary metabolites in large scale. Moreover, cell culture must be addressed to produce active secondary metabolites. For chemical analysis, new extraction techniques and chromatographic methods such as HPLC must be used in order to detect new compounds. For antimicrobial tests, standard isolates must be used in order to assure that my negative results are attributed to the use of clinical isolates. Moreover, other organic solvents like methanol and butanol must be used in order to know if there is a relationship between the solvent type and antimicrobial activity of the extract. Traditionally, *C. spinosa* is used to treat rheumatism. The anti-inflammatory effect of caper extracts should be investigated by using animal models.

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Appendix

Appendix (A)

§ Appendix table 1: Analysis of variance (Kruskal-Wallis ANOVA) for the effect of BA concentration on number of leaves for shoot Multiplication from *C. spinosa* nodal segments.

Comparing Multiple Independent Samples			
Sample size	Sum of Ranks		
MS Free	10	334	
MS + 0.5 mg/L IAA + 1.0 mg/L GA3	10	314.5	
MS + 0.5 mg/L IAA + 1.0 mg/L GA3 + 0.5 mg/L BA	10	344.5	
MS + 0.5 mg/L IAA + 1.0 mg/L GA3 + 1.0 mg/L BA	10	326	
MS + 0.5 mg/L IAA + 1.0 mg/L GA3 + 1.5 mg/L BA	10	232.5	
MS + 0.5 mg/L IAA + 1.0 mg/L GA3 + 2.0 mg/L BA	10	278.5	
H	2.915082	N	60
Degrees Of Freedom	5	p-level	0.713076
H (corrected)	3.338758		
Median Test			
Overall Median	0.5	Chi-square	6.4
p-level	0.269219		

§ Appendix table 2: Analysis of variance (Kruskal-Wallis ANOVA) for the effect of BA concentration on shoot height for shoot Multiplication from *C. spinosa* nodal segments.

Comparing Multiple Independent Samples			
Sample size	Sum of Ranks		
MS Free	10	396	
MS + 0.5 mg/L IAA + 1.0 mg/L GA3	10	358	
MS + 0.5 mg/L IAA + 1.0 mg/L GA3 + 0.5 mg/L BA	10	304	
MS + 0.5 mg/L IAA + 1.0 mg/L GA3 + 1.0 mg/L BA	10	301.5	
MS + 0.5 mg/L IAA + 1.0 mg/L GA3 + 1.5 mg/L BA	10	220	
MS + 0.5 mg/L IAA + 1.0 mg/L GA3 + 2.0 mg/L BA	10	250.5	
H	6.983115	N	60
Degrees Of Freedom	5	p-level	0.221899
H (corrected)	8.12657		
Median Test			
Overall Median	0	Chi-square	6
p-level	0.306219		

§ Appendix table 3: Analysis of variance (ANOVA) for the effect of Zeatin on number of leaves for shoot Multiplication from *C. spinosa* nodal segments.

Summary				
Groups	Sample size	Sum	Mean	Variance
MS Free	10	43	4.3	219
MS+ 0.5 mg/L Zeatin	10	66	6.6	582
MS+ 1.0 mg/L Zeatin	10	39	3.9	251
MS+ 2.0 mg/L Zeatin	10	50	5	338
Total	40		4.95	10.5102

ANOVA							
Source of Variation	d.f.	SS	MS	F	p-level	F crit	Omega Sqr.
Between Groups	3	42.5	14.1666	1.3881	0.262	2.8662	0.0282
Within Groups	36	367.4	10.2055				
Total	39	409.9					

Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	-2.3	1.609883735	0.1157004	rejected
1 vs 3	0.4	0.27997978	0.7810115	rejected
1 vs 4	-0.7	0.489964615	0.6269759	rejected
2 vs 3	2.7	1.889863515	0.0664225	rejected
2 vs 4	1.6	1.11991912	0.2697769	rejected
3 vs 4	-1.1	0.769944395	0.4460931	rejected

§ Appendix table 4: Analysis of variance (ANOVA) for the effect of Zeatin on shoot height for shoot Multiplication from *C. spinosa* nodal segments.

Summary				
Groups	Sample size	Sum	Mean	Variance
MS Free	10	12.2	1.22	16.82
MS+ 0.5 mg/L Zeatin	10	12.4	1.24	16.38
MS+ 1.0 mg/L Zeatin	10	8.4	0.84	11.18
MS+ 2.0 mg/L Zeatin	10	10.9	1.09	14.55
Total	40		1.0975	0.275635

ANOVA							
Source of Variation	d.f.	SS	MS	F	p-level	F crit	Omega Sqr.
Between Groups	3	1.01675	0.338917	1.25357	0.304825	2.866266	0.018663
Within Groups	36	9.733	0.270361				
Total	39	10.74975					

Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	-0.02	0.086009	0.931911	rejected
1 vs 3	0.38	1.634167	0.110483	rejected
1 vs 4	0.13	0.559057	0.579403	rejected
2 vs 3	0.4	1.720176	0.093536	rejected
2 vs 4	0.15	0.645066	0.52276	rejected
3 vs 4	-0.25	1.07511	0.289105	rejected

§ Appendix table 5: Analysis of variance (T- Test) for the effect of basal media types on callus induction from leaf segments of *C. spinosa* under dark.

Comparing Means [With T-Test Assuming Equal Variances (homoscedastic)]			
Descriptive Statistics			
VAR	Sample size	Mean	Variance
MS + 0.5 mg/L 2,4-D	12	105.025	512.55
B5 + 0.5 mg/L 2,4-D	12	69.575	115.76
Summary			
Degrees Of Freedom	22	Hypothesized Mean Difference	0
T-criter. value	4.89	Pooled Variance	314.16
Two-Tailed Distribution			
P(T<=t)Probability, corresponding to Student criterion)	0.0001	Critical Value (5%)	2.07
One-Tailed Distribution			
P(T<=t)Probability, corresponding to Student criterion)	0.0000	Critical Value (5%)	1.71

§ Appendix table 6: Analysis of variance (ANOVA) for the effect of different 2,4-D concentrations on callus induction from leaf segments of *C. spinosa* in dark.

Summary				
Groups	Sample size	Sum	Mean	Variance
MS+ 0.0 mg/L 2,4-D	12	344.4	28.7	10390.7
MS+ 0.1mg/L 2,4-D	12	552.9	46.075	25560.59
MS+0.5 mg/L 2,4-D	12	2606.3	217.1916	580554.61

ANOVA							
Source of Variation	d.f.	SS	MS	F	p-level	F crit	Omega Sqr.
Between Groups	2	260447.65	130223.83	284.97045	0	3.28	0.94
Within Groups	33	15080.112	456.97308				
Total	35	275527.76					

Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	-17.375	1.9909261	0.0543412	rejected
1 vs 3	-188.49167	21.598444	0	accepted
2 vs 3	-171.11667	19.607518	0	accepted

§ Appendix table 7: Analysis of variance (ANOVA) for the effect of different media types on *C. spinosa* callus growth in dark.

Summary				
Groups	Sample size	Sum	Mean	Variance
0.5 mg/L 2,4-D	12	1671	139.25	243042.76
1.0 mg/L 2,4-D	12	1977.6	164.8	353221.16
0.5 mg/L 2,4-D + 1.0 mg/L BA	12	2667.6	222.3	618181.86
1.0 mg/L 2,4-D + 1.0 mg/L BA	12	2421.7	201.80833	508821.29
1.0 mg/L 2,4-D + 1.5 mg/L BA	12	3267.2	272.26667	895046.28
Total	60		200.085	3665.64

ANOVA							
Source of Variation	d.f.	SS	MS	F	p-level	F crit	Omega Sqr.
Between Groups	4	127831.17	31957.793	19.8738	0.0	2.5396	0.5571
Within Groups	55	88441.746	1608.0317				
Total	59	216272.92					

Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	-25.55	1.560699	0.12413	rejected
1 vs 3	-83.05	5.073036	4.45E-06	accepted
1 vs 4	-62.5583	3.821321	0.00033	accepted
1 vs 5	-133.017	8.125206	4.26E-11	accepted
2 vs 3	-57.5	3.512337	0.000877	accepted
2 vs 4	-37.0083	2.260621	0.027619	rejected
2 vs 5	-107.467	6.564507	1.69E-08	accepted
3 vs 4	20.49167	1.251715	0.215786	rejected
3 vs 5	-49.9667	3.05217	0.003448	accepted
4 vs 5	-70.4583	4.303885	6.67E-05	accepted

§ Appendix table 8: Analysis of variance Two-Way ANOVA for the effect of different types of cytokinins together with different light conditions on *C. spinosa* callus growth.

Descriptive Statistics				
Dependent Variable: weight				
Light condition	**Cytokinin types**	**Mean**	**Std. Deviation**	**N**
dark	Zeatin	256.3313	79.92967	16
	2ip	166.9125	54.87576	16
	TDZ	192.8688	67.17962	16
	BA	285.2188	81.3475	16
	Total	225.3328	84.72031	64
light	Zeatin	208.5938	87.51984	16
	2ip	192.2063	23.26848	16
	TDZ	207.9938	68.43811	16
	BA	148.85	80.89114	16
	Total	189.4109	72.29627	64
Total	Zeatin	232.4625	85.94035	32
	2ip	179.5594	43.40721	32
	TDZ	200.4313	67.15011	32
	BA	217.0344	105.675	32
	Total	207.3719	80.48876	128

Tests of Between-Subjects Effects						
Dependent Variable: weight						
Source	Type III Sum of Squares	df	Mean Square	F	Sig.	Partial Eta Squared
Corrected Model	223378.3	7	31911.18571	6.38	2.15	0.27
Intercept	5504396.101	1	5504396.101	1102.01	2.56	0.91
condition	41292.19531	1	41292.19531	8.26	0.005	0.06
cytokine	49427.54563	3	16475.84854	3.29	0.023	0.07
condition * cytokine	132658.5591	3	44219.51969	8.85	0	0.18
Error	599383.6788	120	4994.86399			
Total	6327158.08	128				
Corrected Total	822761.9788	127				
a	R Squared = .271 (Adjusted R Squared = .229)					

§ Appendix table 9: Analysis of variance (Kruskal-Wallis ANOVA) for the effect of auxins types on root length during adventitious roots induction from leaf segment of *C. spinosa*.

Comparing Multiple Independent Samples			
Sample size	Sum of Ranks		
MS + 0.1 mg/L NAA	16	1782	
MS + 0.5 mg/L NAA	16	1403	
MS + 1.0 mg/L NAA	16	1854.5	
MS + 0.1 mg/L IBA	16	1295.5	
MS + 0.5 mg/L IBA	16	1064	
MS + 1.0 mg/L IBA	16	1225	
MS + 0.1 mg/L IAA	16	1064	
MS + 0.5 mg/L IAA	16	1064	
MS + 1.0 mg/L IAA	16	1064	
MS Free	16	1064	
H	24.25529	N	160
Degrees Of Freedom	9	p-level	0.003915
H (corrected)	55.32064		
Median Test			
Overall Median	0	Chi-square	100.5
p-level	0		

§ Appendix table 10: Analysis of variance (Kruskal-Wallis ANOVA) for the effect of auxins types on number of roots during adventitious roots induction from leaf segments of *C. spinosa*.

Comparing Multiple Independent Samples			
Sample size	Sum of Ranks		
MS + 0.1 mg/L NAA	16	1771.5	
MS + 0.5 mg/L NAA	16	1404	
MS + 1.0 mg/L NAA	16	1875	
MS + 0.1 mg/L IBA	16	1291	
MS + 0.5 mg/L IBA	16	1064	
MS + 1.0 mg/L IBA	16	1218.5	
MS + 0.1 mg/L IAA	16	1064	
MS + 0.5 mg/L IAA	16	1064	
MS + 1.0 mg/L IAA	16	1064	
MS Free	16	1064	
H	24.67536	N	160
Degrees Of Freedom	9	p-level	0.003352
H (corrected)	56.34913		
Median Test			
Overall Median	0	Chi-square	100.5
p-level	0		

§ Appendix table 11: Analysis of variance (Kruskal-Wallis ANOVA) for the effect of different carbon sources on number of roots during adventitious roots induction from leaf segment of *C. spinosa.*

Comparing Multiple Independent Samples			
Sample size	Sum of Ranks		
Sugar Free	12	456	
Sucrose (30g/L)	12	663	
Sucrose (15g/L)	12	456	
Fructose (30g/L)	12	581	
Fructose (15g/L)	12	502	
Sorbitol (30g/L)	12	456	
Sorbitol (15g/L)	12	456	
H	5.6271709	N	84
Degrees Of Freedom	6	p-level	0.4662216
H (corrected)	19.538623		
Median Test			
Overall Median	0	Chi-square	59.666667
p-level	0		

§ Appendix table 12: Analysis of variance (Kruskal-Wallis ANOVA) for the effect of different carbon sources on root length during adventitious roots induction from leaf segment of *C. spinosa*.

Comparing Multiple Independent Samples			
Sample size	Sum of Ranks		
Sugar Free	12	456	
Sucrose (30g/L)	12	663.5	
Sucrose (15g/L)	12	456	
Fructose (30g/L)	12	582.5	
Fructose (15g/L)	12	500	
Sorbitol (30g/L)	12	456	
Sorbitol (15g/L)	12	456	
H	5.6838235	N	84
Degrees Of Freedom	6	p-level	0.4595233
H (corrected)	19.72008		
Median Test			
Overall Median	0	Chi-square	59.666667
p-level	0		

§ Appendix table 13: Analysis of variance (Kruskal-Wallis ANOVA) for the effect of different treatments of auxins in semi-solid media on root growth area.

Comparing Multiple Independent Samples			
Sample size	Sum of Ranks		
MS Free	12	262	
MS + 1.0 mg/L NAA	12	412	
MS + 2.0 mg/L NAA	12	487.5	
MS + 1.0 mg/L NAA + 1.0 mg/L IBA	12	256.5	
MS + 2.0 mg/L NAA + 1.0 mg/L IBA	12	412	
H	11.4209	N	60
Degrees Of Freedom	4	p-level	0.02222
H (corrected)	12.84133		
Median Test			
Overall Median	0.5	Chi-square	14.66667
p-level	0.005445		

§ Appendix table 14: Analysis of variance (Kruskal-Wallis ANOVA) for the effect of different plant source (*ex* and *in vitro* leaf segment) and different NAA concentrations (1.0 and 2.0 mg/L) on root growth area in semi-solid media.

Comparing Multiple Independent Samples			
Sample size	Sum of Ranks		
MS + 2.0 mg/L NAA (*in vitro*)	12	351	
MS + 1.0 mg/L NAA (*in vitro*)	12	342.5	
MS + 2.0 mg/L NAA (*ex vitro*)	12	237.5	
MS + 1.0 mg/L NAA (*ex vitro*)	12	245	
H	4.7595663	N	48
Degrees Of Freedom	3	p-level	0.1902735
H (corrected)	5.3404537		
Median Test			
Overall Median	0.75	Chi-square	3.3333333
p-level	0.3430301		

§ Appendix table 15: Analysis of variance (T- Test) for the effect of NAA at different concentrations (1.0 and 2.0 mg/L) in liquid media on root growth. The increase in root growth area during six weeks of culture was assayed.

F-Test Two-Sample for Variances				
Descriptive Statistics				
VAR	Sample size	Mean	Standard Error (of Mean)	Variance
2.0 mg/L NAA (difference: from starting to 6 weeks of culture)	6	3	0.428174	1.1
1.0 mg/L NAA (difference: from starting to 6 weeks of culture)	6	4.083333	1.126696	7.616667
Summary				
F-crit. value	0.14442	P(F<=f)(Probability, corresponding to Fisher criterion)	0.026681	

Comparing Means [With T-Test Assuming Equal Variances (homoscedastic)]			
Descriptive Statistics			
VAR	Sample size	Mean	Variance
1.0 mg/L NAA (difference: from starting to 6 weeks of culture)	6	4.083333	7.616667
2.0 mg/L NAA (difference: from starting to 6 weeks of culture)	6	3	1.1
Summary			
Degrees Of Freedom	10	Hypothesized Mean Difference	0
T-criter. value	0.898799	Pooled Variance	4.358333
Two-Tailed Distribution			
P(T<=t)Probability, corresponding to Student criterion)	0.389889	Critical Value (5%)	2.228139
One-Tailed Distribution			
P(T<=t)Probability, corresponding to Student criterion)	0.194944	Critical Value (5%)	1.812461

§ Appendix table 16: The name of chemicals and reagents and their company name and catalog number which were used during the study.

Name	**Company**	**Cat. No.**
Acetic acid glacial	Frutarom	5550020
Agare (Blood)	Oxoid	719671
Agar (MacConkey)	Oxoid	41671
Agar (Mueller Hinton)	Oxoid	41699
Agar (Potato dextrose)	Neogen	101.814A
Agar European Bacteriological	Hy labs	5090076
Ampicillin Antimicrobial Susceptibility Test Discs (10µg)	Oxoid	3B
6-Benzyleaminopurine	Duchefa biochemie	B0904.0005
Chloroform	Frutarom	5551020
2,4-Dichlorophenolxyacetic acid	Sigma	125k0703
Dimethyl Sulfoxide (DMSO)	aMResco	2587B005
Etheyl alcohol (absolute)	Frutarom	5551640
D-Fructose	Duchefa biochemie	F0801.1000
Gamborg B5 medium including vitamins	Duchefa biochemie	G0210.0010
Gentamycin Antimicrobial Susceptibility Test Discs (10µg)	Oxoid	24 B
Gibberellic acid	Duchefa biochemie	60907.0005
Indole-3-acetic acid	Duchefa biochemie	10901.0025

Indole-3-butyric acid	Sigma	105k1151
Isopentenyl adenine	Duchefa biochemie	2365-40-4
McCowns woody plant basal salt mixture	Sigma	047k2329
Methyl Alcohol (Methanol)	Frutarom	5552390
Murashige and skoog medium including vitamin	Duchefa biochemie	M0222.0050
1-Naphtalene acetic acid	Duchefa biochemie	N0903.0025
D-Sorbitol	Biological industries	S0807
Sulphamethoxazole/ trimethoprim Antimicrobial Susceptibility Test Discs (25µg)	Oxoid	52B
Sucrose	Duchefa biochemie	S0809.1000
Thidiazuron	Duchefa biochemie	51707-55-2
Tris base	Promega	H5131
Zeatin	Duchefa biochemie	16.37.36-4

§ Appendix table 17: The exact weight of grape vinegar which presents in 1.0 ml.

Plate Weight (g)	**Plate No.**				
	Plate 1	**Plate 2**	**Plate 3**	**Plate 4**	**Plate 5**
Empty plate weight	55.1399	59.5252	58.9151	59.7535	55.7334
Plate final weight	56.0837	60.4719	59.8590	60.7027	56.6854
Vinegar weight in 10 ml	0.9438	0.9467	0.9439	0.9492	0.9520
Average weight in 10 ml		0.94712			
Vinegar weight in 1.0 ml		0.0947			

Appendix (B)

1. Preparing of Extract Samples for Antimicrobial Assay

- 200 mg of *C. spinosa* (root bark, leaves, and callus) extracts which were extracted by different solvents (apple vinegar, grape vinegar, and absolute ethyl alcohol), were dissolved in 1.0 ml DMSO (200mg/ml).
- The extracts and DMSO were mixed well and centrifuged for 2.0 min at 5000 rpm.
- In order to have different concentrations of extracts, serial dilution of 200 mg/ml stock solution was done by using 50 mM Trise-HCL Buffer. 500 µl from 200 mg/ml extract stock solution were added to 500 µl of 50 mM Trise-HCL, the solution was mixed well and then 500 µl of this solution were added to 500 µl of 50 mM Trise-HCL and so on.
- Different extracts concentrations were obtained; 100 mg/ml, 50 mg/ml and 25 mg/ml.
- All extracts were sterilized by using membrane filters (pore size 0.45 µm) before adding to disks.
- The same procedures were done on pure apple and grape vinegar, DMSO, and distilled water in order to be used as blank (negative control).

2. Procedure of Standard Disc Diffusion Method

- Cultures were prepared by transferring one loop of stock bacteria and fungi which are kept in –80ºC and streaked onto culture media (MacConkey Agar media for *e-coli* and Sheep Blood Agar media for other microorganisms) to obtain isolated colonies.
- These cultures incubated in 37ºC overnight and subcultures were obtained by transferring one colony from this 18 hr incubated cultures to fresh culture media.
- After incubation at 37°C overnight, 1 or 2 well-isolated colonies were selected with an inoculating loop, and transferred to a tube of sterile saline (≈ 2 ml) and vortex thoroughly. The bacterial suspension should then be compared to the 0.5 McFarland standard and the fungal suspension to 1.0 McFarland standard.

- If the bacterial or fungal suspension does not appear to be the same density as the McFarland 0.5 and the McFarland 1.0 respectively, turbidity can be reduced by adding sterile saline or increased by adding more bacterial, fungal growth.
- A sterile cotton swab was dipped into the suspension. Then pressed firmly against the inside wall of the tube just above the fluid level, the swab was rotated to remove excess liquid.
- Inoculated culture was dispersed by streaking the sterile swab over the entire sterile agar surface (Muller Hinton Agar for bacteria and Potato Dextrose Agar for fungi) by rotating the plate 60^{o} each time to ensure the inoculum uniformly spread. Lastly, swab all around the edge of the agar surface (Figure B1).

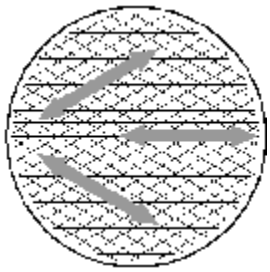

Figure B1: Uniform inoculation of culture onto agar surface, plate should be swabbed over the entire surface of the medium three times, rotating the plate 60 degrees after each application from Boppet al., (1999), p.65.

- The inoculated plates were allowed to sit for 5-10 minutes to let the broth absorb into agar.
- Standard size blank Whatman filter paper discs (6.00 mm. in diameter) sterilized by autoclaving and dried at 60ºC for 1 hour, were saturated with the tested extracts (20µl of extract).
- Four concentrations of each extract types were used in this test, 200 mg/ml, 100 mg/ml, 50 mg/ml, and 25 mg/ml.
- For control discs, 20 µl of (DMSO, distilled water, apple and grape vinegars) were added to sterilized filter discs separately.
- The discs were air dried at room temperature to remove any residual solvent which might interfere with the determination.
- Standard antibiotic discs of Ampicillin, Gentamicine, and Sulphamethoxazole/ trimethoprim were used for positive control.
- The discs were placed on each plate (4 to 5 discs per 25 ml plate) and then gently pressed to ensure contact with gar surface.

- Plates were incubated for 24hr at 37 ^{0}C for bacterial strains and 48h for fungi.
- At the end of the incubation period the antibacterial activity was evaluated by measuring the inhibition zone by using a ruler and results were expressed as mm. The zone of inhibition was considered as an indicator for the antimicrobial activity.
- After all steps plates were sterilized and discarded properly as biohazard material.