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

In Vitro Propagation of *Crataegus aronia* L. and Secondary Metabolites Detection

By

Wala' Shuaib Al- Manasrah

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

July, 2012

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

Crataegus aronia L., Arabic name "Zaroor", commonly known as hawthorn tree, is a deciduous shrub found in eastern Mediterranean region and it is distributed mainly on dry hillsides and the mountainous regions. In the past few decades, populations of *C. aronia* are subjected to genetic erosion and classified as endangered species as many other wild plants in Palestine due to severe habitat fragmentation, over-exploitation, extensive agricultural and human activities, overgrazing, and premature harvest by local people. All these factors lead to the rarity of many wild plants and accelerated their extinction in many areas. Besides the misuse of *C. aronia* plant, efforts to propagate hawthorn by conventional horticultural techniques are problematic. Seed show slow and low germination percentage and mature tree cuttings are difficult to root. Therefore, plant tissue culture may offer a good alternative technique to propagate and conserve the endangered plant species. In the present work, the major objectives were to optimize an effective micropropagation protocol for the purpose of conservation and to detect the presence of phenolic compounds from different tissue sources. After the stony endocarp was removed and the seed coat was scratched, micropropagation of *C. aronia* was initiated and seeds were cultured on water-agar media or water-agar media supplemented with 0.5 mg/L GA_3. Addition of 0.5 mg/L GA_3 doesn't affect germination percentage which reached about 100% on the two media. *In vitro* shoot proliferation was tested with kinetin, BA, and zeatin at the levels (0.0, 0.5, 1.0, 2.0 mg⁄L). Culture of microshoots on QL media supplemented with 2.0 mg⁄L zeatin and 0.05 mg/L IAA gave the highest proliferation results (1.75 shoots/explant). For *in vitro* rooting, QL media supplemented with 30 g⁄L sucrose and IBA, IAA, and NAA at the levels (0.0, 0.5, 1.0, 1.5 mg⁄L), did not induce adventitious roots at any concentration. Callus was initiated from different explants with percentage ranged from 13- 100% on B5 and MS media supplemented with 0.5 mg/L 2,4-D under light and dark condition. Different concentration of TDZ, BA and NAA were used to test callus growth. The highest callus fresh weight was obtained

on B5 or MS media supplemented with 1.0 mg/L TDZ and 0.5 mg/L NAA for cotyledonary and leaves callus. TLC analysis from aceton:water (70:30) v/v extracts, of *in vitro* and *ex vitro* vegetative parts, detected the presence of phenolic compounds as major components. MS analysis indicated the presence of phenolic compound with molecular weight similar to chlorogenic acid (353.3).

Key words: *Crataegus aronia* L., Callus, Secondary Metabolites, Extraction, Plant Conservation

ملخص بالعربية

التكثير الدقيق داخل الانابيب و الكشف عن المركبات الثانوية من *Crataegus aronia* L.

ولاء شعيب المناصرة

شجرة الزعرور شجرة صغيرة متساقطة الاوراق و النوع *aronia* ينتشر على نطاق واسع في منطقة شرق البحر الابيض المتوسط في المناطق الجبلية الجافة. في العقود القليلة الماضية اعتبر الزعرور واحدا من النباتات المهددة بالانقراض في فلسطين بسب الظروف القاسية التي يتعرض لها النبات بسب الرعي الجائر و بعض الممارسات الزراعية و البشرية الخاطئة بالإضافة الى صعوبة الانبات باستخدام طرق الزراعة التقليدية. ويعود ذللك الى انخفاض نسبة انبات البذور و بطء نموها إضافة الى صعوبة تجذير افرع من الشجرة الناضجة. تم اللجوء الى تقنيات تكثير النبات داخل المختبر بواسطة التكثير الدقيق حيث تعتبر بديل جيد لتكثير النباتات المهددة بالانقراض، بالإضافة الى امكانية استغلالها لإنتاج مركبات ثانوية مفيدة. تهدف الدراسة الحالية الى وضع بروتوكول لزراعة و تكثير نبات الزعرور، بالإضافة الى تأسيس بروتوكول لإنتاج الكالوس و تنميته من اجزاء مختلفة من النبات، ايضا تم اجراء تحاليل نوعية للكشف عن مركبات الفينول في اجزاء مختلفة من النبات. تم بنجاح تأسيس نبات الزعرور داخل الانابيب من البذور. حيث تم كسر غلاف الثمرة الصلب و بعد ذلك تم خدش غلاف البذرة و من ثم زرعت البذور حيث كانت اعلى نسبة انبات على بيئة مائية صلبة. لم يسجل فرق في نسبة الانبات بعد إضافة منظم النموGA_3 على مستوى 0.5 ملغم/لتر حيث سجل في التجربتين نسبة الانبات تقارب 100%. أعطت بيئة QL مدعمة بمنظم النمو zeatin عند تركيز2.0 ملغم/لتر + IAA عند تركيز 0.05 ملغم/لتر اعلى نسبة تفرع (1.75تفرع/ساق). اما بالنسبة للتجذير فلم يتم استحثاث جذور ثانوية بأي من الاوكسينات IAA, IBA, NAA عند تراكيز (1.5,1.0,0.5)ملغم/لتر. اما الكالوس فقد تم استحثاثه بنجاح من الفلقتين و الاوراق الحقيقية والجذور على نوعين من الوسط الغذائي (MS وB5) المضاف لكل منهما 0.5 ملغم/لتر 2,4-D وذلك في الضوء و في الظلام. ايضا تم دراسة تاثير منظمات النمو TDZ او BA مع NAAعلى وزن الكالوس حيث سجل الهرمونين TDZعلى تركيز 1.0 ملغم/لتر بالاضافة ال NAA على تركيز 0.5 ملغم/لتر اعلى وزن رطب. تم باستخدام TLC الكشف عن مركب رئيسي من الفينولات في مستخلص اسيتون : ماء (30:70) من الاوراق التي نمت في الأنابيب والتي جمعت من الطبيعة و الكالوس المجفف. تم فصل المركب الفينولي و اظهر MS ان هذا المركب ذو كتلة جزيئية تساوي 353.3 غم/مول و هذه الكتلة المولية مشابهة للكتلة المولية للمركب الفينولي "chlorogenic acid".

الكلمات المفتاحية: الزعرور، الكالوس، المواد الثانوية، الاستخلاص.

DECLARATION

I declare that the Master Thesis entitled "***In Vitro* Propagation of *Crataegus aronia* L. and Secondary Metabolites Detection"** 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: Wala' Shuaib Al-Manasrah

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Dedication

To My Husband for his support, encouragement, and patience all the times.

To My Children (Sadeel and Tariq), thanks for being the happiness of my life.

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 Uncle Abu Tariq and his family, for their encouragement and support.

Wala'

Acknowledgement

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

I also wish to express my warm and sincere gratitude to my co-advisor Dr. Rami Arafeh 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 Ghada Abu Khalaf

Big thanks and best wishes to all of you

Wala' Shuaib Al-Manasrah

Abbreviations:

Word or sentence	Abbreviation
2,4-Dichlorophenoxyacetic acid	2,4-D
6-(4-Hydroxy-3-methylbut-2-enylamino) purine	zeatin
6-Furfurylaminopurine	kinetin
Analysis of variance	ANOVA
And other	et al.
And so on	etc.
Benzyl adenine	BA
Centimeter	cm
Completely Randomized Design	CRD
Degree centigrade Celsius	°C
For example	i.e.
Gamborg's B5	B5
Gibberellic acid	GA_3
Glutathione	GSH
Gram	g
Gram per liter	g/L
High Performance Liquid Chromatography	HPLC
Hydrochloric acid	HCL
Indole-3-acetic acid	IAA
Indole-3-butyric acid	IBA
Infra Red	IR
Iron (III) Chloride	$FeCl_3$
Isopentenyl adenine	2ip
Least Significant Difference	LSD
Liter	L
Mass Spectrometry	MS
McCown woody plants media	Mcc
Meter	m
Microliter	µL
Milligram per kilogram	$mgkg^{-1}$

Milligram per litter	mg/L
Milliliter	ml
Minute	min
Molar	M
Murashige and Skoog salt mixture	MS
Naphthaleneacetic acid	NAA
Nuclear Magnetic Resonance	NMR
Page	P
Photosynthetic Photon Flux Density	PPFD
Plant Growth Regulator	PGR
polyvinylpyrrolidone	PVP
Potassium hydroxide	KOH
Pound per square inch	Psi
Quoirin and Lepoivre salt mixture	QL
Rounds per minute	rpm
Sample size	N
Second	sec
Standard mean error	SE
Sterile Distilled Water	SDW
Thidiazuron	TDZ
Thin Layer Chromatography	TLC
Ultra Violet	UV
Volume by volume	v/v
Weight by volume	w/v
World Health Organization	WHO

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CHAPTER 1

Introduction

Herbal remedies and alternative medicine are used throughout the world, and in the past, herbs were the only original source of most drugs. Additionally, there is an ongoing world-wide revolution which is mainly premised on the belief that herbal remedies are less damaging to the human body due to mild or reversible side effects than synthetic drugs (Elango et al., 2009; Shatoor, 2011). According to the World Health Organization (WHO) about 80% of the world population relies on traditional medicine for primary health care and more than 30% of the plant species have been used medicinally (WHO, 2008). In fact, herbs or plants are the oldest friends of mankind. Besides food and shelter, they also serve the humanity with treatment of different ailments. Recently, medicinal plants provided the modern medicine with numerous therapeutic agents (Evans, 2002 cited in Hudiab et al.,2008).

In Palestine, there are about 2780 plant species found on its small area. Among them there are more than 700 are renowned for their uses as medicinal herbs (Ali-Shtayeh et al., 2008). According to the study conducted by Said et al. (2002), around 129 plant species are still in use in the treatment of various human health problem including liver, skin, respiratory, digestive diseases, and cancer. Many of wild plants are becoming endangered due to rural and urban extension, illegal collection and uncontrolled deforestation, industrial pollution, and low level of awareness in the Palestinian population (PIALES, 1996). The problem is worsened by the continued extension of Israeli settlements and the separation wall (UAWC, 2010). Moreover, many ethnopharmacological studies that were conducted in the Middle Eastern area emphasized on the fact that many of plants used in traditional medicine are now rare or endangered due to the ongoing destruction of their natural habitat, overharvesting of wild species, and detrimental climatic and environmental changes (Azaizah et al., 2006; Al-Qura'n, 2009). To counter the overexploitation of natural resources and consequent threats to biodiversity, alternative techniques in biotechnology could be implemented. The advancements in biotechnological methods of culturing plant

tissues and cells provided new means for conserving and rapid propagation of valuable, rare and endangered plant species.

Crataegus aronia L., or its Arabic name "Zaroor" is a known medicinal plant in Palestine. It contains mixture of flavonoids mainly oligomeric procyanidine, which is responsible for the medicinal activity. Furthermore, several ethnobotanical and ethnopharmacological surveys on the therapeutic uses of *C. aronia* have revealed that it has been used in Arab traditional medicine to treat rheumatism, diabetes, digestive system, urinary system and stones, cardiovascular diseases, cancer, and sexual weakness (Ali-Shtayeh et al., 2000; Said et al., 2002; Aburjai et al., 2007).

In Palestine and during the past few decades, populations of *C. aronia* are facing genetic erosion and the plant is classified as endangered species as many other wild plants due to severe habitat fragmentation, over-exploitation, extensive agricultural and human activities, overgrazing, and premature harvest by local people. All these factors lead to the rarity of many wild plants and accelerated their extinction in many areas (ROTEM, 2002; BERC, 2002). Besides the misuse of *C. aronia* plants, efforts to propagate *Crataegus* in general by conventional horticultural techniques proved problematic in both seed germination which is slow and of low percentage. Moreover, mature cuttings are difficult to root and rarely succeed (Nokes, 2001 cited in Maharik et al., 2009a). All of the previous reasons raise the risk of losing genetic resources of the plant.

Based on this background, there are strong challenges to conserve this plant by implementing alternative techniques used in the conservation of endangered species (Fay, 1992). *In vitro* propagation techniques would enable the conservation of endangered *C. aronia* plants and the production of valuable secondary metabolites such as polyphenolics compound in a high quantity via *in vitro* plant.

1.1 Overview of Species

Hawthorn is a common name of all plant species in the genus *Crataegus* that belongs to the Rosaceae family. In this genus, there are around 280 species (Bahorun et al., 2003; Al-Abdallat et al., 2011) distributed across different temperate region of both hemispheres, including the Mediterranean region, North Africa, Europe and Central Asia and Eastern North America (Khalil, 2008; Lo et al., 2009; Bor et al., 2012). *Crataegus aronia* (synonym for *Crataegus azarolus* L.) is the main hawthorn species found in the eastern Mediterranean region and it is distributed mainly on dry hillsides

and in mountainous regions (Froehlicher et al., 2009; Al-Abdallat et al., 2011; Shatoor, 2011).

Hawthorn is a small shrub or spreading tree with thorny branches, three-to five-lobed deciduous green leaves, white flowers, and red, yellow-green, or yellow fruits (Dai et al., 2007). Normal size trees can reach a height of up to 10 m. However average hawthorn trees have height between 2 to 5 m (Ozcan et al., 2005;Yanar et al., 2011). Most species of hawthorn have prominent, long, straight and sharp thorn, ranging from 1-5 inches (Khalil, 2008; Shatoor, 2011). The hawthorn tree prefers the forest margins of lower and warmer areas (Ozcan et al., 2005). Fruits ripen in late summer and are highly attractive to birds, which consume the fruit and disperse the seeds (Yanar et al., 2011).

Many hawthorn species are grown for their edible fruits in Asia, Central America, and various Mediterranean countries. They are also used for landscaping and other ornamental purposes or as frost-resistant rootstocks for pear and apples (Maharik et al., 2009a; Al-Abdallat et al., 2011). Moreover, Chinese hawthorns (*Crataegus pinnatifida*) is a commercially important tree whose fruit is used for making jam, juice, and sweets (Kami et al., 2009; Liu et al., 2010a). In addition hawthorn species are listed as herbal drugs in pharmacopoeias due to their high contents of flavonoides, oligomeric proanthocyaninidins, and ethanobotanical and ethanopharmacological compounds (Kami et al., 2009; Al-Abdallat et al., 2011)

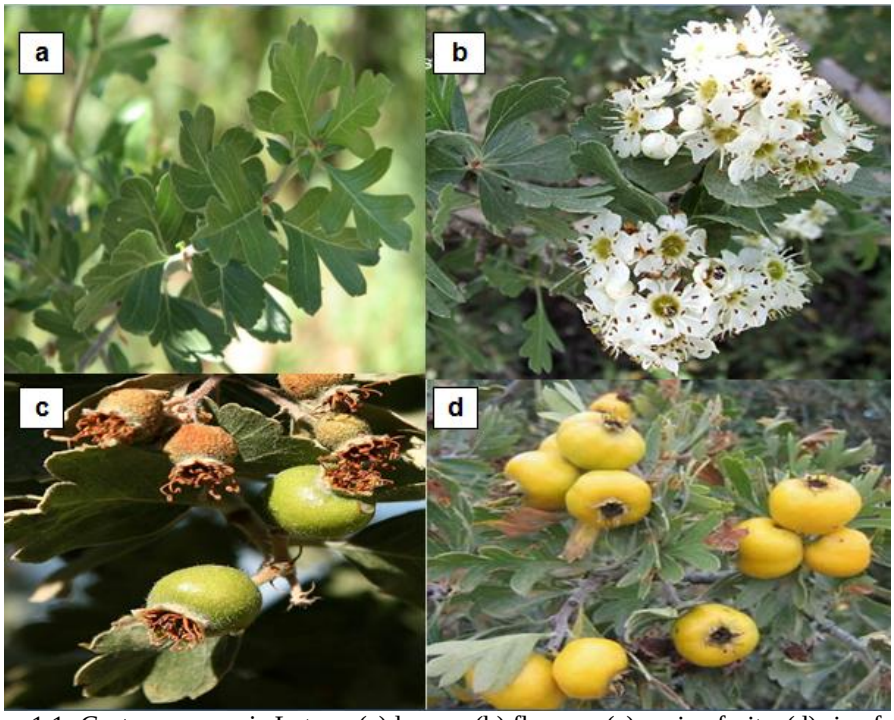

Figure 1.1: *Crataegus aronia* L. tree: (a) leaves, (b) flowers, (c) unripe fruits, (d) ripe fruits.

Many independent studies indicated that the medicinal use of extract or tinctures prepared from the leaves, fruits, or flowers of plant species from the genus *Crataegus* dates back to ancient times (Bahorun et al., 2003; Ljubuncic et al., 2005; Liu et al., 2010b). Nowadays, the plant is mainly used to treat cardiovascular diseases and is considered to be generally safe and well-tolerated (Ljubuncic et al., 2005; Zick et al., 2009; Luo et al., 2009). The underlying mechanisms whereby *Crataegus* extracts exert their beneficial consequence on cardiovascular function are based on results obtained from *in vitro* and *in vivo* laboratory investigations. The studies showed that the extracts (1) reduce blood pressure and total plasma cholesterol (Bahorun et al., 2003; Khalil, 2008; Liu et al., 2010a), (2) reduce mortality after ischemia reperfusion (Bahorun et al., 2003; Froehlicher et al., 2009), (3) endothelium-dependent vasorelaxation (Vierling et al., 2003; Ling et al., 2008; Liu et al., 2010a), (4) improvement of coronary circulation (Zick et al., 2009; Froehlicher et al., 2009; Liu et al., 2010a), (5) enhance myocardial contraction and conductivity (Froehlicher et al., 2009; Ozyurek et al., 2012), (6) lowering the heart rate (Froehlicher et al., 2009), and (7) direct protection to human low density lipoprotein (LDL) from oxidation or indirect protection via maintaining the concentration of α-tocopherol (the major form of vitamin E) in human LDL (Zhang et al., 2001). Besides the *Crataegus* extract effect on the prevention and treatment of cardiovascular diseases, it protects the brain against ischemia-reperfusion. It reduced the brain damage and improved neurological behavior after 24 a hours of reperfusion (Elango et al., 2009). Moreover, many independent studies have been shown that *Crataegus* extracts display strong antioxidant potential (Liu et al., 2008; Luo et al., 2009; Liu et al., 2010b). For example, Sahloul et al. (2009) reported that opened flowers extract of *C. aronia* presented a strong radical-scavenging activity. Additionally, Ljubuncic et al. (2005) reported that leaves and unripe fruit extract of *C. aronia* possess substantial antioxidant potential, also the extract is an efficient scavenger of superoxide radical, increases intracellular glutathione levels and non toxic. Moreover, a study conducted by Shatoor, (2011) indicate that *C. aronia* whole plant aqueous extract has no acute or sub-acute adverse effect when administered at doses up to 2000 mg kg^{-1} body weight in rats tested during the period of observation. Orhan et al. (2007) showed that leaves and berries extract of three *Crataegus* species including *C. aronia, C. monogyna*, and *C. pseudoheterophylla* are highly effective against *Candida albicans* and *Herpes simpex* virus. Furthermore, extract of *C. monogyna* showed antioxidant capacity and

have antimicrobial activity against Gram- (+) more than Gram- (-) bacteria (Proestos et al., 2008).

Crataegus contains a wide range of phytochemical compounds which present in various parts of the plant and responsible for its pharmacological effects. Many studies showed that the chemical constituent of hawthorn consist of sterols, triterpene acids, flavonoids, oligomeric proanthocyandins, organic acids and cardioactive amines (Baughman and Bradley, 2003; Zuo et al., 2006). Other study on *Crataegus pinnatifida* fruits detected forty-two phenolic compounds. Ideain (cyaniding-3-O-galactoside), chlorogenic acid, procyanidin B2, epicatechin, hyperoside (quercetin-3-O-galactoside) and isoquercitrin (quercetin-3-O-glucoside) were identified using UV spectra, mass spectra and reference compounds. In addition, 35 compounds were tentatively identified based on the UV and mass spectra. These compounds were mostly B-type procyanidins and their glycosides, including aglycons of 3 dimers, 3 trimers, 8 tetramers, 4 pentamers, 2 hexamers and 2 hexosides of procyanidins monomers, 7 hexosides of procyanidins dimers, 1 hexosides of procyanidins trimer, etc (Liu et al., 2010a). Most of previous studies on the phytochemicals of *Crataegus* species revealed phenolic compounds as major constituent of extract. The major phenolic compounds that were identified in previous studies include: hyperoside, isoquercitrin, epicatechin, chlorogenic acid, quercetin, rutin and proto catechuic acid (Bahorun et al., 1994; Chang et al., 2001; Bahorun et al., 2003; Khalil, 2008; Sahloul et al., 2009; Froehlicher et al., 2009).

1.2 *In Vitro* Culture and Micropropagation

Plant tissue culture forms an integral part of any plant biotechnology activity. It offers an alternative to conventional vegetative propagation when conventional methods are difficult, expensive, time consuming, and unsuccessful.

Plant tissue culture can be defined as a collection of techniques used to grow plant cell, tissue or organs under sterile conditions on a nutrient culture medium of known composition and controlled environment. The tissue culture technology explores conditions that promote cell division. *In vitro* growth and development of plant is determined by organic substances (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. The plant growth regulators (cytokinins and auxins) regulate the development of organs on parts of plants growing

in vitro. Cytokinins (kinetin, BA, 2ip, or zeatin) stimulate cell division, growth and development. They induce adventitious shoot formation by decreasing apical dominance (Pierik, 1987). Auxins (IAA, IBA, NAA, or 2,4-D) result in cell elongation and swelling of tissues, cell division (callus formation) and the formation of adventitious roots.

Plant tissue culture of medicinal plants has a number of advantages over traditional propagation methods including:

- The opportunity of year round continuous production of medicinal plant under highly controlled conditions.
- Growing plants 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* growing plants are exposed to all sorts of biotic and abiotic factors and their constituents may vary accordingly.
- It can eliminate concerns regarding over harvesting of rare or endangered valuable medicinal plants (Briskin, 2007).
- It can produce pathogen-free stock plants or genetically superior clones that cannot be propagated by seeds or whose propagation efficiency is low in conventional vegetative propagation (Tikhomiroff et al., 2002).

On the other hand, the widespread use of micropropagated transplants is still limited by high production costs, frequently attributed to a low growth rate, a significant loss of plants *in vitro* due to microbial contamination, poor rooting, low percent survival at the *ex vitro* acclimatization stage and high labor costs.

There are four critical stages for successful micropropagation of plantlets. The first stage, the preparative stage, or stated as phase zero. It involves the correct pretreatment of the starting plant material to guarantee they are disease free as far as possible. The second phase is the establishment of clean starting tissue for aseptic culture and growth. It involves sterilizing protocol to produce aseptic tissues. These aseptic tissues are used for the next stage for shoot multiplication, which can be carried out in a number of ways. Generally, plant growth regulators are used for shoot multiplication. The shoots obtained in phase two are used for root induction at the third phase either *in vitro* or *in vivo*. Finally, at phase four, the *in vitro* plantlets are acclimatized for survival in *ex vitro* condition (Pierik, 1997).

Callus is an amorphous tissue of proliferating and undifferentiated parenchyma cells which frequently occurs in response to wounding at the cut edge of living tissue.

All types of plant organs like 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 not probably match that of the donor plant (Bansal, 2006). The level of plant growth regulators plays a key factor that affects callus formation and growth in the culture medium. The auxin commonly used for callus induction is 2,4-D but NAA and IAA are also used (Afshari et al., 2011). 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 usually subcultured to a new fresh media for further growth. When subcultured regularly, callus will exhibit S-shape or sigmoidal pattern of growth during each passage and for many species subculture period ranges from three to six weeks (Chawla, 2007).

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

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

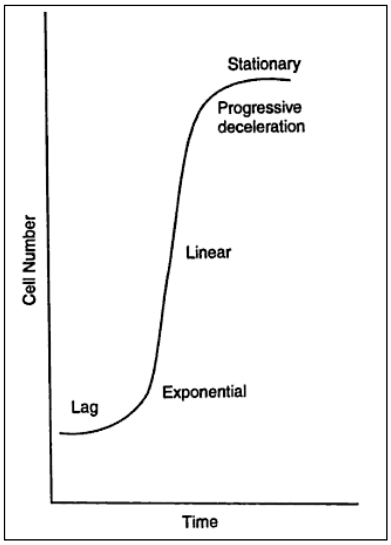

Figure 1.2: A model curve presenting cell number per unit volume of culture to time in a batch grown cell suspension culture (Chawla, 2007), p. 58.

Callus and cell suspension cultures are valuable systems commonly used in the production of secondary metabolites. For hawthorn the first callus culture was reported by Schrall and Becker (1977). *Crataegus monogyna* callus and cell suspension cultures initiated from floral buds were used for the analysis of polyphenols constituents, their production levels and their antioxidant activity (Bahorun et al., 1994, 2003). Hawthorn callus produces mainly proanthocyanidins, B2 dimer, epicatechin, chlorogenic acid and major flavonoid hyperoside (Bahorun et al., 2003; Al Abdallat et al., 2011).

1.3 Extraction of Secondary Metabolites

Secondary metabolites are wide range of chemical compounds from different metabolites families that can be highly inducible in the response of a stress. These compounds are not essential for cell structure and maintenance of life but are often involved in plant protection against biotic or abiotic stresses (Hattenschwiler and Vitousek, 2000; Kennedy and Wightman, 2011). Others, function in: mechanical support, attracting pollinators and fruit dispersers, absorbing harmful ultraviolet radiation, or reducing the growth of nearby competing plants (Kashani et al., 2012).

Pharmaceutical extraction methods involve the separation of medicinally active portions of plant tissues from the inactive components by using selective solvents. During extraction, solvents distribute into the solid plant material and solubilize compounds with similar polarity (Tiwari et al., 2011).

The general techniques of medicinal plant extraction include infusion, maceration, percolation, decoction, digestion, hot continuous extraction (Soxhlet), aqueous-alcoholic extraction by fermentation, counter-current extraction, microwave-assisted extraction, ultrasound extraction (sonication), supercritical fluid extraction, and phytonic extraction (with hydrofluorocarbon solvents) (Tiwari et al., 2011). The properties of good extraction solvent includes, low toxicity, ease of evaporation at low temperature, promotion of fast physiologic absorption of the extract, preservative action, and inability to cause the extract to complex or dissociate (Tiwari et al., 2011).

The basic parameters influencing the quality of an extract are: plant part used as starting material, solvent used for extraction and extraction process (Tiwari et al., 2011; Kashani et al., 2012). While the variations in different extraction methods that will influence quantity and composition of secondary metabolite of an extract depends upon: type of extraction, time of extraction, temperature, nature of solvent, solvent concentration and polarity (Tiwari et al., 2011).

Extracting secondary metabolite from *in vitro* growing plant material has some advantages over *ex vitro* plant including: a) production can be simpler, more reliable, and more predictable, b) separation of the phytochemical can be rapid and efficient, when compared to extraction from complex whole plants, c) interfering compounds that occur in the field-grown plant can be eliminated in cell cultures, d) tissue and cell cultures can yield a source of defined standard phytochemicals in large scale volumes, e) tissue and cell cultures are a potential model to investigate elicitation, g) cell cultures can be radio labeled, such that the accumulated secondary products, when provided to laboratory animals as feed, can be traced metabolically (Collin, 2001; Karuppusamy, 2009).

1.4 Thin Layer Chromatography

Chromatography is an analytical method consists of combined techniques that are widely used for the separation, isolation, identification, and quantification of components in a mixture. Chromatography is defined as a process for separating compounds in a mixture that contains a large number of organic compounds; separation is based on differences in migration rates among the sample components. Components of the mixture are carried through the stationary phase by the flow of a mobile phase (Fried and Sherma, 1994).

Thin layer chromatography (TLC) is a qualitative analysis of extracts that serves as one of the many methods in providing a chromatographic plant extract fingerprint (Wagner and Bladt, 2009). TLC has some advantages over other chromatography methods because it is simple, quick, and inexpensive procedure that can be used for the analysis of plant extracts (Robards and Antolovich, 1997).

TLC is a tool of liquid chromatography where the sample is applied as a small spot of concentrated aqueous plant extract to the origin of a thin sorbent layer such as alumina, cellulose powder, or silica gel supported on a glass, or metal plate. This layer consists of finely divided particles and constitutes the stationary phase. While the mobile phase is a solvent or a mixture of organic and/or aqueous solvents in which the spotted plate is placed. The mobile phase moves through the stationary phase by capillary action (Fried and Sherma, 1994). Plates can be visualized, depending on the compounds chemical structure at visible light, UV-254 nm and 365 nm or by using spray reagents, phenols can detect at UV-254 (Wagner and Bladt, 2009).

1.5 Mass Spectrometry

Mass spectrometry (MS) is a powerful analytical technique which is used to identify unknown compounds, to quantify known compounds, and to illuminate the structure and chemical properties of molecules (Pan and Raftery, 2007). Detection of compounds can be accomplished with very minute quantities. Combining MS with nuclear magnetic resonance (NMR) techniques provide unequivocal structural information for the individually isolated compounds. However, a much smaller amount of material is needed for MS techniques. The conventional way of studying natural products includes fractionation of a crude mixture or extract, separation and isolation of the individual components using liquid chromatography and structure elucidation using various spectroscopic methods (UV, IR, NMR, MS) (Exarchou et al., 2005).

1.6 Polyphenolic Compounds

Most of previous studies showed that the pharmacological effects of *Crataegus* have mainly been attributed to the polyphenolic contents (Bahorun et al., 1994; Chang et al., 2001; Bahorun et al., 2003; Khalil, 2008; Sahloul et al., 2009; Froehlicher et al., 2009).

Phenolic compounds are ubiquitously found across the plant kingdom, with about 10,000 structures identified to date (Kennedy and Wightman, 2011). Phenolic compounds are chemically defined by the presence of at least one aromatic ring bearing one hydroxyl substituent (phenols) or more than one hydroxyl (polyphenols), including their functional derivatives such as esters and glycosides. Polyphenols can be roughly divided into two groups: (1) low molecular weight compounds [e.g. simple phenols (C_6), phenolic acids (C_6–C_1) and flavonoids (C_6–C_3–C_6)] and (2) oligomers and polymers of relatively high molecular weight (e.g. tannins) (Hattenschwiler and Vitousek, 2000; Kennedy and Wightman, 2011). Polyphenols in plants derive mainly from the shikimic acid pathway through aromatic carboxylic acids (Hattenschwiler and Vitousek, 2000; Matkowski, 2008). Other references showed that they derive from phenylpropanoid biosynthetic pathway, in which phenylpropanoid is a product of shikimic acid pathway (Duthie et al., 2003; Kennedy and Wightman, 2011). They are vital to the physiology of plants, being involved in various functions such as lignification and structure, pigmentation, pollination, pathogen and predator resistance, allelopathy, and growth (Duthie et al., 2003).

Recently, it has become clear that certain polyphenolic products have considerable antioxidant ability since they possess the ideal chemical structure for scavenging free radical. Polyphenolic have redox properties which allow them to act as reducing agents, hydrogen donators, singlet oxygen quenchers, and they also have metal chelation properties (Hakkim et al., 2008; Proestos et al., 2008). Furthermore, plant phenolics can act as chain-breaking antioxidants inhibiting lipid oxidation, thus affecting beneficially food deterioration during storage and processing (Kylli et al., 2010). In this connection, it has been demonstrated that *in vitro* they are more active than vitamins E and C (Zdunczyk et al., 2002; Olthof et al., 2003; Sokol Letowska et al., 2007). Furthermore, numerous *in vitro* studies indicate that plant polyphenols can potentially affect diverse processes in mammalian cells that, if also occurring *in vivo*, could have anti-carcinogenic and anti-atherogenic implications. These processes include gene expression, apoptosis, platelet aggregation, blood vessel dilation, intercellular signaling, P-glycoprotein activation and the modulation of enzyme activities associated with carcinogen activation and detoxification (Duthie et al., 2000).

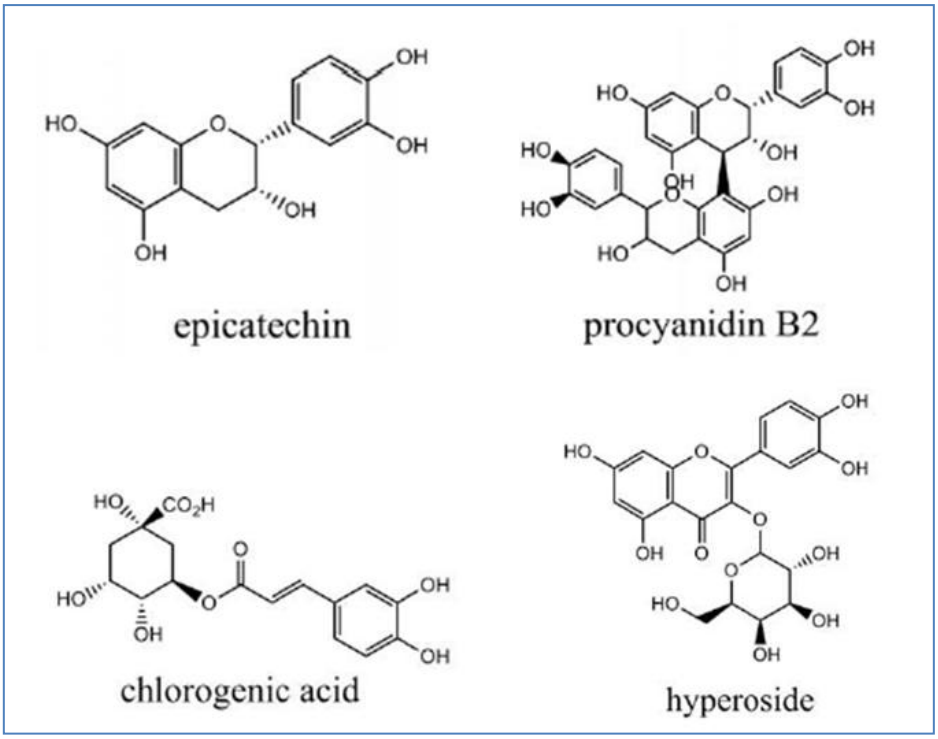

Figure 1.3: Chemical structures of some polyphenolic compounds

Among polyphenolic compounds that are present in some hawthorn species extract is chlorogenic acid. It has a very pronounced antioxidant activity due to the presence of a high number of hydroxyl groups and a carboxyl moiety (Matkowski, 2008). Chlorogenic acids are a family of esters formed between certain trans-cinnamic acids and quinic acid, which are present in the human diet from coffee beverages, vegetables and fruits. They have anti-bacterial, anti-mutagenic, antioxidant and many other biological activities (Valls et al., 2007). Moreover, they can control glucose metabolism by modulating glucose-6-phosphatase activity *in vivo* (Valls et al., 2007). In the current study the major phenolic compound that was isolated and characterized by TLC, $FeCl_3$ staining, and by MS spectroscopy with molecular weight of 353.3 g/mole which is similar to the molecular weight of phenolic compound chlorogenic acid. Further study is needed to confirm the structure.

CHAPTER 2

PROBLEM STATEMENT AND OBJECTIVES

2.1 Problem statement

C. aronia is subjected to genetic erosion and considered as endangered plant species as many other wild plants in Palestine, (ROTEM, 2002; BERC, 2002). Besides the misuse of *C. aronia* plant, efforts to propagate it by conventional horticultural techniques proved problematic either in germinating seeds which is slow and of low percentage or cuttings which are difficult to root and rare to succeed (Nokes, 2001 cited in Maharik et al., 2009). *C. aronia* tree is a valuable medicinal plant that has a high antioxidant content of phenolic compounds. Those reasons raised the risk of losing the genetic resources of *C. aronia*, therefore, we believe there is an urgent need to maintain this species by using highly advanced tissue culture methods that are efficient. The *in vitro* propogation of *C. aronia* may offer a new hope for the conservation of endangered plants like *C. aronia*. Moreover, this technique may enhance the production of valuable secondary metabolites such as polyphenolic compounds with known medicinal record.

2.2 Objectives

The main objectives of the present study are:

- Conservation of *C. aronia* in Palestine through massive propagation by tissue culture
    1. Optimizing a suitable growth medium and optimal concentration of growth regulators for culture initiation, shooting, and rooting.
    2. To determine appropriate growth medium for callus induction and maintenance from different tissues.
- To explore major phenolic compound in extract of , *in vitro* leaves, *ex vitro* leaves and callus

CHAPTER 3

MATERIALS AND METHODS

The experimental work in this study was conducted in the Plant Tissue Culture Laboratory in the Biotechnology Research Center at the Palestine Polytechnic University, Hebron, Palestine.

3.1 Source of Plant Material

The *in vitro* culture in this study was carried out from seeds. Seeds were collected in October 2008 from wild plant in "Alaroub Agricultural Station" , 7.0 Km north of Hebron. After removing the fruit bulb, endocarps were dried in shade at room temperature and kept in a closed jar.

3.2 Chemicals and Reagents for *In Vitro* Culture

All basal salts for plant growth, plant growth regulator (PGRs), and other reagents were purchased from Duchefa Biochemie and Sigma-Aldrich chemical companies unless stated otherwise. All chemical and reagents with Cat. number are listed in appendix 21.

3.3 Media Preparation and Sterilization

The growth medium was prepared by using one of the basal salts of MS, B5, QL or Mcc. The recommended salt weight for every medium in addition to 3.0 % (w/v) sucrose were dissolved in distilled water up to 50 % of the final volume. The needed PGRs (in mg bases) 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. Finally media was solidified with 0.06 % (w/v) agar. All components were heated with continuous stirring until they completely dissolved. Finally, each medium was dispensed in autoclavable media bottles for use in sterile polystyrene Petri dishes. Media in other experiments including seedlings was poured in 15x150 mm test tubes (8.0 ml) or in 250.0 ml Erlenmeyer flasks (50.0 ml). Flasks were closed with aluminum foil and test tubes were closed with autoclavable snap caps. Media was autoclaved at 121°C and 15 Psi pressure for 20 min.

3.4 Surface Sterilization of Plant Material

Under the laminar air-flow cabinet, seeds were immersed in 100 ml of 20% (v/v) commercial bleach "Clorox" (5% sodium hypochlorite) for 20 min with continuous shaking on an orbital shaker at 110 rpm. Then, seeds were washed with sterile deionized water (SDW) 3 times with shaking (2 min each). Finally seeds were washed with 70% (v/v) ethanol solution for 30 sec. Then rinsed with SDW 3 times (2 min. each).

3.5 Growth Conditions

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

3.6 Types of Cultured Explants and Mother Stock Establishment

3.6.1 *In Vitro* Seed Germination

Seeds were extracted from the woody fruit endocarp then surface sterilized. After sterilization and before germination (Under the laminar air-flow cabinet) the seed coat was scratched with a blade. Sterilized seeds of *C. aronia* were inoculated on the surface of agar media (first treatment), and agar media supplemented with 0.5 mg/L GA_3 (second treatment). The seeds were cultured in 5.0 cm Petri-dishes which were filled with 8.0 ml of media. Eleven replicates with five seeds per replicate were used in each media. The plates were kept in growth room under light condition.

Figure 3.1: (a) The fruit woody endocarp where seeds are inside , (b) Cracking the stony endocarp to extract the seeds, (c) *C. aronia* seeds.

3.6.2 *In Vitro* Multiplication of Mother Stock Plants

For *in vitro* multiplication of mother stock of *C. aronia*, three types of media were tested; (a) full strength MS medium, (b) full strength Mcc medium, and (c) full strength QL medium. The three types of media were supplemented with 0.5 mg/L

zeatin to promote shooting. After four weeks of inoculating seeds on the germination media, primary microshoots appeared and 0.5 cm long of these microshoots were subcultured in 15x150 mm test tubes that were filled with 8.0 ml media. Each treatment composed of ten test tubes as replicates. The tubes were kept in growth room under 16 h light (PPFD = 40-45 μmoles/m^2/sec) /8h dark at (24 ± 1 ºC).

3.6.3 Micropropagation

3.6.3.1 Shoot Multiplication

To examine the optimum medium for shoot proliferation**,** microshoots were cultured in Erlenmeyer flasks (250 ml) containing 50.0 ml of the media listed in table 3.1:

Table 3.1: Treatments that were used for testing shoot proliferation.

Treatment No.	Medium type	PGRs
1	MS	0.2 mg/L kinetin + 0.05 mg/L IAA
2	Mcc	0.2 mg/L kinetin + 0.05 mg/L IAA
3	QL	0.2 mg/L kinetin + 0.05 mg/L IAA

Microshootes were obtained from seedlings that were initiated on agar medium supplemented with 0.5 mg /L GA_3. After one month when the true leaves appeared, cotyledons were removed and seedlings of about 0.6 cm, six leaves, and one bud were transferred to the tested media. Four replicates with three seedlings per replicate were used in each treatment.

3.6.3.2 Shoot Proliferation on QL Media

Based on the result observed in section 3.6.3.1, microshooots (0.6 cm) were subcultured in Erlenmeyer flask (250.0 ml) containing 50.0 ml of full strength QL media supplemented with 0.05 IAA and different concentration of cytokinins. Media listed in table 3.2.

Table 3.2: Treatments that were used for shoot proliferation which consisted of different types and concentrations of cytokinins supplemented to QL media.

Medium No.	PGRs Type and Concentrations
1	0.2 mg/L Kinetin + 0.05 IAA mg/l
2	1.0 mg/L Kinetin + 0.05 IAA mg/l
3	2.0 mg/L Kinetin + 0.05 IAA mg/l
4	0.5 mg/L BA + 0.05 IAA mg/l
5	1.0 mg/L BA + 0.05 IAA mg/l
6	2.0 mg/L BA + 0.05 IAA mg/l
7	0.5 mg/L Zeatin + 0.05 IAA mg/l
8	1.0 mg/L Zeatin + 0.05 IAA mg/l
9	2.0 mg/L Zeatin + 0.05 IAA mg/l

Four replicates with three microshoots per replicate were used in each treatment. Data were reported after 6 weeks of subculture for the number of proliferated shoots, shoot height, number of leaves/ explants. Callus and the plant performance were monitored and recorded.

3.6.3.3 *In Vitro* Rooting

In vitro rooting was experimented by subculturing microshoots (0.6 cm) in Erlenmeyer flask (250.0 ml) containing 50.0 ml of QL media supplemented with 30.0 g/L sucrose and one of the PGRs NAA, IBA, or IAA at the concentrations (0.5, 1.0, or 1.5 mg/L). Each treatment composed of four flasks as replicates with three seedlings per replicate. Data were reported after 6 weeks for the number of roots, root length and shoot height for each treatment.

3.6.4 Callus Induction and Culture

From the *in vitro* growing material callus was induced from three explant sources; cotyledons, true leaves, and roots. This part of experiment was shared for all callus types .

3.6.4.1 Effect of Basal Media and Light on Callus Induction

Callus was induced from *in vitro* growing cotyledons, true leaves and roots. Cotyledon and true leaves were cut into pieces of 4.0 mm^2. Roots were cut into pieces of 1.0 cm length. Each explant was inoculated on 5.0 cm Petri-dishes filled with 8.0

ml of the needed media. Each Petri-dish was cultured with four explants. Two types of basal media were tested;

1) Full strength MS medium supplemented with 0.5 mg/L 2,4-D under light and dark conditions.
2) Full strength B5 medium supplemented with 0.5 mg/L 2,4-D under light and dark conditions.

Each treatment has ten replicates, five in light and five under dark conditions.

3.6.4.2 Effect of Basal Media and Light on Callus Growth

To determine the optimum basal salt for callus growth, induced callus clumps from cotyledons, true leaves and roots of known weight were subcultured on full strength MS and B5 media supplemented with 0.5 mg/L 2,4-D. Each treatment has eight Petri-dishes (5.0 cm) as replicate, each cultured with four explants. Four replicates in light and four replicates under dark condition.

3.6.4.3 Estimation of Callus Growth Curve

To estimate the growth curve for the three callus types four callus clumps of known initial weight were cultured on full strength MS or B5 media supplemented with 0.5 mg/L 2,4-D in 18 Petri-dishes (5.0 cm).

a) Growth curve for callus induced from cotyledons: four callus clumps of known initial weight (average initial weight of about 190.0 mg) were cultured on full strength B5 medium supplemented with 0.5 mg/L 2,4-D in 18 Petri-dishes (5.0 cm).
b) Growth curve for callus induced from true leaves: four callus clumps of known initial weight (average initial weight of about 130.0 mg) were cultured on full strength MS medium supplemented with 0.5 mg/L 2,4-D in 18 Petri-dishes (5.0 cm).
c) Growth curve for callus induced from roots: four callus clumps of known initial weight (average initial weight of about 250.0 mg) were cultured on full strength B5 medium supplemented with 0.5 mg/L 2,4-D in 18 Petri-dishes (5.0 cm).

Callus fresh weight in three plates was recorded weekly for five or six weeks.

3.6.4.4 Callus Growth and Subculture

To determine the optimum medium for callus growth, induced callus clumps were subcultured on full strength basal salt; B5 for callus from cotyledons, and MS for callus from leaves each supplemented with different concentrations and combinations of TDZ and NAA or BA and NAA as listed in table 3.3.

Table 3.3: Treatments that were used for testing cotyledonary and leaves callus growth.

Treatment No.	TDZ (mg/L)	NAA (mg/L)	BA (mg/L)	Hormone Combinations
1	0.1	0.1	0	0.1 mg/L TDZ + 0.1 mg/L NAA
2	0.5	0.1	0	0.5 mg/L TDZ + 0.1 mg/L NAA
3	0.5	0.5	0	0.5 mg/L TDZ + 0.5 mg/L NAA
4	1.0	0.5	0	1.0 mg/L TDZ + 0.5 mg/L NAA
5	0	0.1	0.1	0.1 mg/L BA + 0.1 mg/L NAA
6	0	0.1	0.5	0.5 mg/L BA + 0.1 mg/L NAA
7	0	0.5	0.5	0.5 mg/L BA + 0.5 mg/L NAA
8	0	0.5	1.0	1.0 mg/L BA + 0.5 mg/L NAA

Three Petri dishes (10.0 cm) in each treatment were used, each was cultured with eight explants and kept under light. The average starting weight of callus was 240.0 mg and data was recorded after four weeks.

3.6.5 Effect of Basal Media on Red Colored Callus

Callus was induced from cotyledons on full strength B5 media supplemented with 0.5 mg/L 2,4-D under dark conditions for four weeks, then callus clumps were transferred to light and subcultured on the induction media each for four weeks. After six months under light condition, callus clumps were observed to produce red pigment. Two media types were tested in addition to the induction media:

1) Full strength B5 media supplemented with 0.5 mg/L 2,4-D (induction media).
2) Full strength B5 media supplemented with 2.0 mg/L 2,4-D and 1.5 mg/L kinetin.
3) Full strength MS media supplemented with 2.0 mg/L 2,4-D and 1.5 mg/L kinetin.

In each treatment three Petri-dishes (10.0 cm) were cultured by eight callus clumps. The average starting weight of callus was (470.0) mg and data were recorded after four weeks for the callus fresh weight, texture and color.

3.6.6 Effect of Media with Antioxidant on Cotyledonary Callus and Red Colored Callus Growth

Callus that was induced from cotyledons become necrotic after 30 to 40 days, also red colored callus get browning after 35 to 40 days and the red pigment disappeared gradually. Accordingly, four antioxidant treatments were performed to minimize phenolic browning during callus multiplication:

1) 1.0 g/L polyvinylpyrrolidone (PVP) was added to full strength B5 media supplemented with 0.5 mg/L 2,4-D.
2) 2.0 g/L active charcoal was added to full strength B5 media supplemented with 0.5 mg/L 2,4-D.
3) 150.0 mg/L citric acid was added to full strength B5 media supplemented with 0.5 mg/L 2,4-D.
4) 100.0 mg/L ascorbic acid was added to full strength B5 media supplemented with 0.5 mg/L 2,4-D.

Four Petri-dishes, (5.0 cm) were cultured in each treatment by four callus clumps. The average starting weight of callus induced from cotyledons was 220.0 mg and that of red colored callus was 300 mg. Data was recorded after four weeks for the callus fresh weight and color.

3.7 Extraction of Secondary Metabolites

3.7.1 Plant Material

Four sources of *C. aronia* tissues used; leaves from *ex vitro* growing plants, *in vitro* leaves, callus induced from leaves and callus induced from cotyledons. Leaves from *ex vitro* were collected from the wild near Bani Naim village, 7.0 km east of Hebron in June 2011. 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. *In vitro* leaves were grown for 6 weeks on Mcc supplemented with 0.5 mg/L zeatin and air-dried in shade at room temperature four five to seven days. Callus induced from leaves and callus induced from cotyledons of one and half year old were grown on MS media supplemented by 0.5 mg/L 2,4-D for four weeks and dried in the drying oven at 40ºC for 24 hours. Each of the above samples was powdered at the extraction day by a blender.

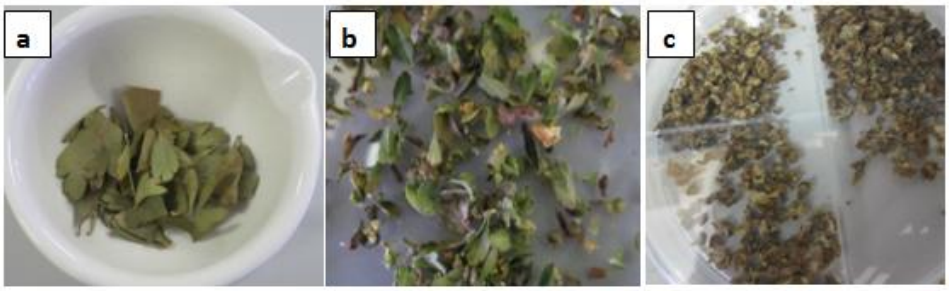

Figure 3.2: *C. aronia* plant materials which were used in the extraction step. (a) *ex vitro* leaves, (b) *in vitro* leaves, (c) dried callus.

3.7.2 Chemicals and Reagents for Extraction and TLC analysis

For extraction and TLC analysis, acetone, chloroform, methyl alcohol, and glacial acetic acid were purchased from Frutarom chemical company. Iron (III) chloride ($FeCl_3$) was purchased from Sigma-Aldrich.

3.7.3 Extraction Procedure

Powdered plant materials of 3.0 g dry weight from each of: leaves from *ex vitro* growing plants, *in vitro* leaves, callus induced from leaves and callus induced from cotyledons were dissolved in 100 ml acetone/water (70:30) (v/v). The mixture container was covered with aluminum foil and continuously stirred with magnetic stirrer at room temperature. After 96 hours, supernatant was removed by centrifugation for 15 min at 5000 rpm. Solvents were air dried under the fume hood, the percentage yield of dry extracts was calculated in each treatment. The dried extract was stored at -20°C after acetone/water was completely evaporated.

3.7.4 Sample Preparation for TLC Analysis

Samples for major compound detection were prepared as follow, 100.0 mg of dried extracts of the previous plant material were dissolved in 1.0 ml acetone/water (70:30) (v/v) solvent, and were shaken for 24 h, centrifuged for 2 min at 5000 rpm, and the supernatant was taken for further analysis.

3.7.5 TLC Analysis and Phenolic Compounds Assay

For qualitative detection of phenolic compounds, TLC analysis was performed using precoated TLC plates (Merck, Germany Silica gel 60 F254, 0.25 mm) as stationary phase. Different combination and ratio of different solvents were evaluated as mobile phase for proper separation, and chloroform:acetone:acetic acid (60:18.5:21.5 v/v/v)

was selected for TLC detection and separation. Plates were run three times in the mobile phase, after each run, the plates were air-dried. Aromatic substances were detected using UV light detector at 254 nm. Plates were sprayed with $FeCl_3$ solution (1.0 g of $FeCl_3$ dissolved in 100.0 ml water:methanol (50:50)) for visualization of phenolic substances, and heated until spots were brown, grey or black. R*f* value of the major compound was measured by using the ratio referred to

$$R_f = \frac{distance\ spot\ moves}{distance\ solvent\ moves}.$$

3.7.6 Separation and characterization of the Major Compound

Silica gel chromatography was used for detection and purification of major phenolic compound from *in vitro* leaves extracts. Sample was loaded on plate chromatography using chloroform:methanol (10:1 v/v) as mobile phase, before solvent reached the end, plate was removed and air-dried, silica gel with the desire phenolic compound was scratched from the plate, suspended in chloroform:methanol (10:1) and filtered. Solvents were evaporated at room temperature under fume hood and dried sample was send to mass spectroscopy for molecular weight identification.

3.7.7 ESI-MS (Infusion) with Bruker EsquireLC

ESI mass spectra were performed on a *Bruker ESQUIRE-LC* quadrupole ion trap instrument (*Bruker Daltonik GmbH,* Bremen, Germany), equipped with a combined Hewlett-Packard Atmospheric Pressure Ion (API) source (*Hewlett-Packard Co.*, Palo Alto, CA, USA). The solutions (about 0.1-1 µmol/ml) were continuously introduced through the electrospray interface with a syringe infusion pump (*Cole-Parmer* 74900-05, *Cole-Parmer Instrument Company*, Vernon Hills, Illinois, USA) at a flow rate of 5 µl min-1. The MS-conditions were: Nebulizer gas (N2) 15 psi, dry gas (N2) 7 l/min, dry temperature 300°C, capillary voltage 4000 V, end plate 3500 V, capillary exit 100 V, skimmer1 30 V, and trap drive 70. The MS acquisitions were performed at normal resolution (0.6 u at half peak height), under ion charge control (ICC) conditions (10'000) in the mass range from *m/z* 100 to 2000.

3.8 Experimental Design and Statistical Analysis

All experiments were set 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, data that not normally distributed, the non-parametric test of Kruskal-Wallis ANOVA was carried out.

CHAPTER 4

RESULTS AND DISCUSSION

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

4.1.1 *In Vitro* Seed Germination

Successful *in vitro* establishment of *C. aronia* was achieved in this study. Seeds of *C. aronia* start to germinate after six days (Figure 4.1). After 4 weeks the percentage of germination was reported in the following table.

Table 4.1: The seed germination percentages as observed in different media after four weeks of culture. Sample size (n) = 55.

Medium Type	Germination Percentage (%)	Abnormal seedlings percentage (%)
Water-agar	98	5.45
Water-agar + 0.5 mg/L GA_3	100	9.09

Based on that, seeds can be considered a good starting material for the *in vitro* establishment of *C. aronia* species. GA_3 addition has no effects on germination percentage or time that seeds need to germinate. Abnormal seedlings exhibit some form of growth, but have insufficient plant structures to maintain a healthy plant, such as missing roots, apical meristems, or terminal buds.

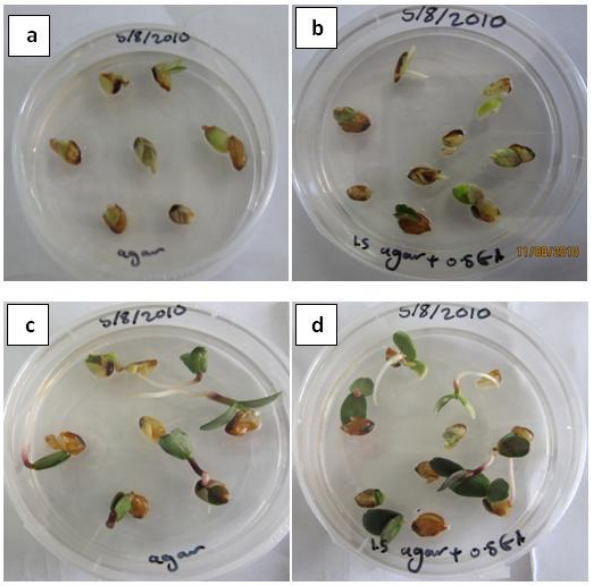

Figure 4.1: Seed germination of *C. aronia*. (a) seed germination after six days on agar media, (b) seed germination after six days on agar + 0.5 mg/L GA_3 media, (c) seed germination after ten days on agar media, (d) seed germination after ten days on agar + 0.5 mg/L GA_3 media.

To the best of our knowledge, no previous studies were conducted to put a protocol for the *in vitro* seed germination of *C. aronia.* However, Maharik et al. (2009a) reported that only 4% of *Crataegus sinaica* -a closely related similar species to *C. aronia*- seeds successfully germinated under *in vitro* conditions. Different treatments to enhance seed germination were conducted in their study. They removed the stones and sown seeds *in vitro* immediately after removing from ripe fruits. Compared to our study, the germination percentage was 100%. According to our observation, cutting the seed coat might played an important role in germination process. All the seeds used in this study were cut before inoculation. In preliminary experiment seeds of non cut coat did not germinate.

4.1.2 *In Vitro* Multiplication of Mother Stock Plants

Culturing mother stock of *C. aronia* in test tubes on three different media types were ineffective and large callus pieces were observed at the bases of explants. Plant growth and development were minimal. 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. Result is reported in Table 4.2.

Table 4.2: Effect of different media type supplemented with 0.5 mg/L zeatin on shoot induction, elongation and plant proliferation of *C. aronia* after four weeks of culture in test tubes. Sample size (n) = 10.

Media type	Number of Leaves ± SE	Number of Shoots ± SE	Shoot Height (cm) ± SE	Callusing Percentage (%)
QL + 0.5 mg/L Z	9.6 ± 1.29	1.0 ± 0.0	0.95 ± 0.14^{b}	70.00
Mcc + 0.5 mg/L Z	11.7 ± 0.83	1.0 ± 0.0	1.53 ± 0.17^{a}	60.00
MS + 0.5 mg/L Z	11.4 ± 0.85	1.0 ± 0.0	1.25 ± 0.18^{ab}	70.00

The significance between treatments was tested by ANOVA, no statistical difference between those media in number of shoots or in number of leaves. Regarding shoot height, Mcc media gave statistical significance when compared to QL but not with MS. Large pieces of callus at the basis of explant were formed in the three types of media. The occurrence of callus on bases of *in vitro* plantltes is supposed to inhibit auxiliary shoot proliferation in woody plant tissue culture (Daffalla et al., 2011). Thus, we decide to culture in flasks and to start from low PGR concentration.

4.1.3 Micropropagation

4.1.3.1 Shoot Multiplication

Microshoots of 0.6 cm length obtained from germinated seeds were used in shoot proliferation on three types of media. The effect of different media on shoot induction, elongation and shoot proliferation was evaluated by measuring the shoot height, number of shoots, and number of leaves after six weeks of culture. Data is summarized in Table 4.3.

Table 4.3: Effect of different media type supplemented with 0.2 mg/L kin + 0.05 mg/L IAA on shoot induction, elongation and plant proliferation of *C. aronia* after six weeks of culture in flasks. Sample size (n) = 12. Different letters within columns showed significant difference at $P = 0.05$ as determined by Fisher's LSD

Media type	**Number of Leaves ± SE**	**Number of Shoots ± SE**	**Shoot Height (cm) ± SE**
MS + 0.2 mg/L kin +0.05 mg/L IAA	12.3 ± 0.78	1.0 ± 0.0	1.16 ± 0.12[a]
Mcc+ 0.2 mg/L kin +0.05 mg/L IAA	11.8 ± 0.99	1.08 ± 0.08	1 ± 0.12 [b]
QL + 0.2 mg/L kin +0.05 mg/L IAA	12.4 ± 0.91	1.4 ± 0.19	0.88 ± 0.08 [a]
Significance test	ANOVA	Kruskal-Wallis ANOVA	ANOVA

According to the number of leaves, shoot height, the significance variations between treatments was tested with analysis of variance by One-Way ANOVA. For the number of shoots the significance between treatments was tested by Kruskal-Wallis ANOVA since the data is not normally distributed. Results showed no statistically significant differences ($p > 0.05$) between the three tested media in number of leaves or in number of shoots. Despite that the ANOVA analysis resulted in non significant difference in number of leaves and number of shoots, QL media supplemented with 0.2 mg/L kinetin and 0.05 mg/L IAA gave the highest number of leaves and shoots. Depending on the previous result, QL media was chosen to carry out shoot proliferation experiment. The present result is compatible with Bhoomsiri and Masomboon (2003) on other species from the family Rosaceae *Rosa damascena* Mill. In which maximum number of shoots were developed on QL media. The QL media positively affected the shoot multiplication of *R. damascena* Mill, and compared to MS media, leading to overall increase in the number of developing shoots. A similar observation was also noted by Bell et al. (2009). They reported for both 'Bartlett' and 'Beurre Bosc' pear cultivars the highest shoot proliferation obtained on QL followed by Driver-Kuniyuki walnut (DKW) and MS medium.

4.1.3.2 Shoot Proliferation on QL Media

Proliferation of shoots was carried out by culturing 0.6 cm microshoots obtained from germinated seeds. Microoshoots were cultured on QL media supplemented with kinetin at 0.2,1.0, 2.0 mg/L, zeatin and BA at 0.5, 1.0, 2.0 mg/L. each treatment was supplemented with 0.05 mg/L IAA. The result of microshoots cultured on previous PGR is shown in table 4.4.

Table 4.4: Effect of different media type supplemented with different PGR on shoot induction, elongation and plant proliferation of *C. aronia* after six weeks of culture in flasks. Sample size (n) = 12.

Medium Type	Number of Leaves ± SE	Number of Shoots ± SE	Shoot Height (cm) ± SE	Callusing Percentage (%)
QL control	7.6 ± 1.0	1.08 ± 0.08	0.78 ± 0.10	0
QL + 0.2 mg/L Kinetin + 0.05 mg/L IAA	12.3 ± 0.91	1.42 ± 0.19	0.88 ± 0.08	0
QL + 1.0 mg/L Kinetin + 0.05 mg/L IAA	8.0 ± 0.81	1.08 ± 0.08	0.6 ± 0.02	0
QL + 2.0 mg/L Kinetin + 0.05 mg/L IAA	7.7 ± 0.92	1.08 ± 0.08	0.67 ± 0.04	0
QL + 0.5 mg/L BA + 0.05 mg/L IAA	8.3 ± 0.88	1.0 ± 0.0	0.68 ± 0.04	33
QL + 1.0 mg/L BA + 0.05 mg/L IAA	10.3 ± 2.03	1.33 ± 0.26	0.88 ± 0.12	17
QL + 2.0 mg/L BA + 0.05 mg/L IAA	11.4 ± 2.51	1.67 ± 0.31	0.88 ± 0.08	17
QL + 0.5 mg/L Zeatin + 0.05 mg/L IAA	9.5 ± 0.99	1.25 ± 0.18	0.9 ± 0.12	67
QL + 1.0 mg/L Zeatin + 0.05 mg/L IAA	10.4 ± 1.03	1.42 ± 0.19	0.93 ± 0.11	67
QL + 2.0 mg/L Zeatin + 0.05 mg/L IAA	12.1 ± 1.23	1.75 ± 0.28	0.99 ± 0.13	50

According to the number of leaves, number of shoots and shoot height, the significance between treatments was tested by Kruskal-Wallis ANOVA since the data is not normally distributed. Results showed statistically significant differences ($p < 0.05$) in number of leaves and in shoot height between the ten tested media. Results indicate no statistical significant in number of shoots. QL medium supplemented with 2.0 mg/L zeatin and 0.05 IAA mg/L was the suitable medium for plant proliferation compared to the other media. The mean number of leaves in this medium was 12.1 ± 1.23 and the mean number of shoots was 1.75± 0.28 and the mean of shoot height was 0.99 ± 0.13. However, QL medium with 0.2 mg/L kinetin + 0.05 IAA mg/L gave the highest number of leaves (12.3 ± 0.91) compared to the other treatments. Despite the non significant in number of shoot QL medium with 2.0 mg/L zeatin and 0.05 IAA mg/L gave the highest number of shoot compared to other media. The differential ability of cytokinins in shoots induction could be attributed to factors such as stability, mobility, and the rate of oxidation and conjugation of the hormones (Daffalla et al., 2011). In general callusing was observed in some flasks, except kinetin containing

flasks. Plants growing in flasks with zeatin hormone exhibit more callusing than BA. While kinetin containing flasks show no callus.

There are several reports on the *in vitro* culture of *Crataegus* species including culture of shoot tips in *Crataegus oxyacantha* Linn. (Rajesh and Bist, 2002), nodal segments in *Crataegus monogyna* (Lapichino and Airo, 2009) callus induction in *Crataegus monogyna* and *Crataegus sinaica* (Bahorun et al., 1994, 2003; Maharik et al., 2009b respectively), and cell suspension culture in *Crataegus monogyna* (Froehlicher et al, 2009). So far, no detailed report is available on the micropropagation of *C. aronia*. In this study, we compared our result with previous studies that have been conducted on other species. No previous report used zeatin hormone for shoot proliferation, instead BA was used and these are some of them.

Rajesh and Bist (2002) reported that higher shoot proliferation of *C. oxyacantha* was attempted by *in vitro* shoot tip culture at 2.0 mg/L BA and 0.02 mg/L IBA on MS medium. Furthermore, Dai et al. (2007) results showed that adventitious buds regenerated from cotyledon leaves grew quickly and 80–100% of buds developed into shoots on B5 medium supplemented with 2.22 μM (= 0.5 mg/L) BA, 2.32 μM (= 0.499 mg/L) Kinetin, and 0.57 μM (= 0.1 mg/L) IAA. A similar observation was recorded by Maharik et al. (2009a); they reported that shoot multiplication from nodal segment explants of *C. sinaica* that initiated from adventitious shoot bud regenerated from cotyledonary leaves increased as BA hormone increased and reached to 100% at 5.0 mg/L of BA on MS medium supplemented with 2.0 mg/L NAA. In the present study, it is concluded that the increasing level of BA or zeatin increased the number of leaves, number of shoots and shoot height in *C. aronia*.

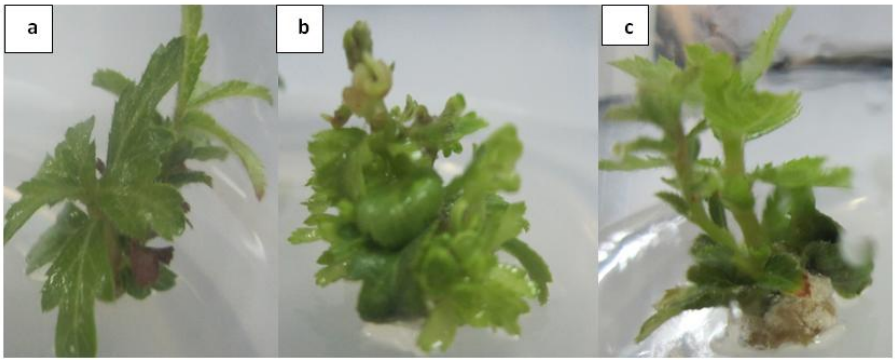

Figure 4.2: *C. aronia* proliferated shoots after six weeks on QL medium supplemented with (a) 2.0 mg/L kinetin and 0.05 IAA mg/L, (b) 2.0 mg/L BA and 0.05 IAA mg/L, (c) 2.0 mg/L zeatin and 0.05 IAA mg/L.

4.1.3.3 *In Vitro* Rooting

To obtain viable plants, *in vitro* growing shoots must have roots in order to be transferred to *ex vitro* conditions. Therefore, growth media was supplemented with different auxins to promote root initiation. In the present study, different auxins like NAA, IBA, and IAA have been used for root induction. By placing microshoots 0.6 cm long on QL medium supplemented with 30 g/L sucrose and 0.0, 0.5, 1.0, or 1.5 mg/L NAA, IBA, or IAA. Microshoots did not give any roots by adding NAA, IBA, or IAA at any of the concentration. No rooting was observed on PGR-free medium as well. The effect of different auxins on number of root, number of shoot, shoot height and number of leaves was evaluated by measuring the shoot height, number of shoots, and number of leaves after six weeks of culture. Data reported in 4.5 Table.

Table 4.5: Influence of NAA, IBA, and IAA levels on number of root, number of shoot, number of leaves, and shoot height of *in vitro* microshoots of *C. aronia.* Sample size (12).

Medium Type	Number of roots	Number of Shoots ± SE	Number of leaves ± SE	Shoot Height (cm) ± SE	Callusing Percentage (%)
QL free (control)	0	1.08 ± 0.08	7.6 ± 1.0	0.78 ± 0.10	0
QL + 0.5 mg/L NAA	0	1.17 ± 0.17	9.5 ± 1.87	0.98 ± 0.14	0
QL + 1.0 mg/L NAA	0	1.17 ± 0.17	10.6 ± 2.32	1.05± 0.22	17
QL + 1.5 mg/L NAA	0	1.0 ± 0.0	7.2 ± 0.51	0.55 ± 0.03	25
QL + 0.5 mg/L IBA	0	1.17 ± 0.17	7.0 ± 0.63	0.58 ± 0.05	0
QL + 1.0 mg/L IBA	0	1.42 ± 0.23	8.2 ± 1.19	0.72 ± 0.08	0
QL + 1.5 mg/L IBA	0	1.0 ± 0.0	7.1 ± 0.83	0.6 ± 0.06	0
QL + 0.5 mg/L IAA	0	1.0 ± 0.0	12.2 ± 1.41	1.71 ± 0.38	0
QL + 1.0 mg/L IAA	0	1.17 ± 0.11	11.1 ± 0.97	1.53 ± 0.29	0
QL + 1.5 mg/L IAA	0	1.0 ± 0.0	9.1 ± 1.22	1.12 ± 0.19	0

Data is not normally distributed. Accordingly, the significance between treatments was tested by Kruskal-Wallis ANOVA. Results show statistically significant differences ($p < 0.05$) in number of leaves and in shoot height between the ten media types. No statistical significant in number of shoots was observed. The absence of rooting in our study is converse with Maharik et al. (2009a) result on *C. sinaica* root induction. They reported that shoots of 0.9 cm length were transferred to either half or full strength MS free hormone medium or supplemented with either 0.5, 1.0, and 2.0 mg/L of IBA or IAA for root development. They showed that only half strength MS medium supplemented with 1.0 mg/L IBA was found to be efficient for roots regeneration of 9% of the shoots that passed elongation stage. Shoots of *C. sinaica* which transferred directly to root induction media without elongation, failed to root.

Elongation stage consists of gradual reduction in BA concentration which caused shoot elongation and induces better rooting. Similar results were also found in *C. pinnatifida* (Chinese Hawthorn) where multiplied shoots required further elongation stage through which shoots were culture on different elongation media containing low concentrations of cytokinins and auxin. Shoots of 2.0 cm of the Chinese hawthorn that were maintained on ½ MS medium containing 2.46 µM (= 0.5 mg/L) IBA failed to root. However, 2.0 cm shoots that incubated on ½ MS medium supplemented with 19.68 µM (= 3.999 mg/L) IBA for 4 days and then transferred to ½ MS medium without auxin addition, about 50% of the shoots rooted successfully (Dai et al., 2007). Maharik et al. (2009a) showed that poor rooting attributed to the long auxin treatment in *C. sinaica*; (the period during which shoots are in a contact with auxin in the root induction medium). This may acts as excessive concentration of auxin that cause inhibition of rooting. However, excessive auxin is frequently characterized by callus formation. In our result no callus formation was detected in rooting experiment except in the case of (1.0, 1.5) mg/L of NAA, the absence of callus at shoot base exclude that any of auxin (IAA, IBA) treatments was supplied in improper high supplement. In this relation it was suggested as a general developmental base that there are at least some cases of non-responsiveness due to the failure of the medium components to elicit competence for induction of organogenesis (Christianson and Warnick, 1988 cited in Maharik et al., 2009a). Moreover it has been found that poor rooting of shoots propagated from field shoot tips of field grown trees limits micropropagation of *Crataegus* species (Gokbuner, 2007 cited in Maharik et al., 2009a). The absence of *in vitro* rooting in this study needs further investigation to obtain viable rooted plant.

4.1.4 Callus Induction and Culture

4.1.4.1 Effect of Basal Media and Light on Callus Induction

After four weeks the percentage of callus induced from cotyledons, leaves, and roots on two basal media under light and dark condition were reported in Table 4.6.

Table 4.6: Percentage of callus induction from cotyledons, true leaves, and roots on two basal media under dark and light conditions after four weeks. Sample size (20).

Explant	% of callus induction in light		% of callus induction in dark	
	B5 + 0.5 2,4-D	MS + 0.5 2,4-D	B5 + 0.5 2,4-D	MS + 0.5 2,4-D
Cotyledons	15	55	50	40
True leaves	13	25	45	30
Roots	100	81	94	94

The callus induction was found to be tissue-dependent. Experimental results showed that roots gave the highest percentage of callus induced on the two basal media under light or dark condition. MS media supplemented with 0.5 mg/L 2,4-D give higher percentage of callus induced in cotyledons and true leaves under light condition. while under dark condition B5 media supplemented with 0.5 mg/L 2,4-D give higher percentage than MS media supplemented with 0.5 mg/L 2,4-D. These differences in callus induction rate might be attributed to the difference in the basal salt formulation and the interaction between the basal media and light condition. It is reported in literature that callus induction is affected by different parameters including: explants, plant growth regulators and light (Afshari et al., 2011).

According to callus texture and color, the callus that was induced from cotyledons and true leaves on MS medium or B5 medium under light condition was compact and gave different colors (red, pink, and green) compared to the one that was induced under dark which was friable and pale yellow in color. Callus which was induced from roots on MS or B5 medium under light or dark conditions was friable in texture. Callus induced from root gave different colors (pink, red, and pale yellow) under light condition while under dark condition gave pale yellow (Figure 4.3). Data is summarized in Table 4.7.

This study demonstrated that the type of basal media and light condition have significant effect on callus color and texture. Afshari et al. (2011) studied the effect of light condition on callus color of *Brassica napus* and they showed that newly initiated callus from cotyledon was creamy in the dark and mostly green in the light. Moreover, they mentioned that light had significant effect on induction of specific enzyme activity which are concerned with the formation of some flavonoid glycoside secondary products. Thus, changing the secondary product profile lead to variation on callus color. Accordingly, different strains of callus can be obtained even from a single explant which varies in features like color, appearance, degree of compaction and morphogenetic potential.

Table 4.7: Color and texture of different callus type from different explants source after induction on MS or B5 medium supplemented with 0.5 mg/L 2,4-D under light and dark condition after four weeks.

CALLUS SOURCE	BASAL SALT TYPE	LIGHT CONDITION	CALLUS COLOR	CALLUS TEXTURE
Cotyledons	MS + 0.5 2,4-D	Light	Different colors; green and red	Compact
	MS + 0.5 2,4-D	Dark	Off white	Friable
	B5 + 0.5 2,4-D	Light	Different colors; green, pink and red	Compact
	B5 + 0.5 2,4-D	Dark	Off white	Friable
True Leaves	MS + 0.5 2,4-D	Light	green	Compact
	MS + 0.5 2,4-D	Dark	pale yellow	Friable
	B5 + 0.5 2,4-D	Light	green	Compact
	B5 + 0.5 2,4-D	Dark	pale yellow	Friable
Roots	MS + 0.5 2,4-D	Light	Different colors; pale yellow, pink and red	Friable
	MS + 0.5 2,4-D	Dark	pale yellow	Friable
	B5 + 0.5 2,4-D	Light	Different colors; pale yellow, pink and red	Friable
	B5 + 0.5 2,4-D	Dark	pale yellow	Friable

In vitro callus induction from different explants was carried out and successfully achieved. The induced callus differs in color and texture. The percentage of callus induction obtained ranges from 13% to 100%. To the best of my knowledge, no previous studies were conducted to put a protocol for callus induction and culture of *C. aronia*. However, few previous studies published on callus induction and culture from other *Crataegus* species. For example Bahorun et al. (1994) induced callus using floral buds from another species; *Crataegus monogyna* cultured on modified B5 Gamborg medium supplemented with 2.0 mg/L 2,4-D and 0.5 mg/L kinetin. On the other hand Maharik et al. (2009b) induced callus using stem and leaf explants of *C. sinaica* on MS medium supplemented with different combinations of 2,4-D and kinetin or NAA and BA in 0.5, 1.0, and 2.0 mg/L concentration for each with a callusing percentage of 100%. Al Abdallat et al. (2011) reported that callus culture was established from internodal stem segments of *C. aronia* incubated on MS medium supplemented with 5.0 mg/L IBA and 0.5 mg/L BA with a callusing percentage of 10%. In comparison to the results of our study, callus was induced from different explants of *C. aronia* on MS or B5 medium supplemented with 0.5 mg/L

2,4-D. The discrepancy between our results and results of previous studies might be attributed to differences in explants type used.

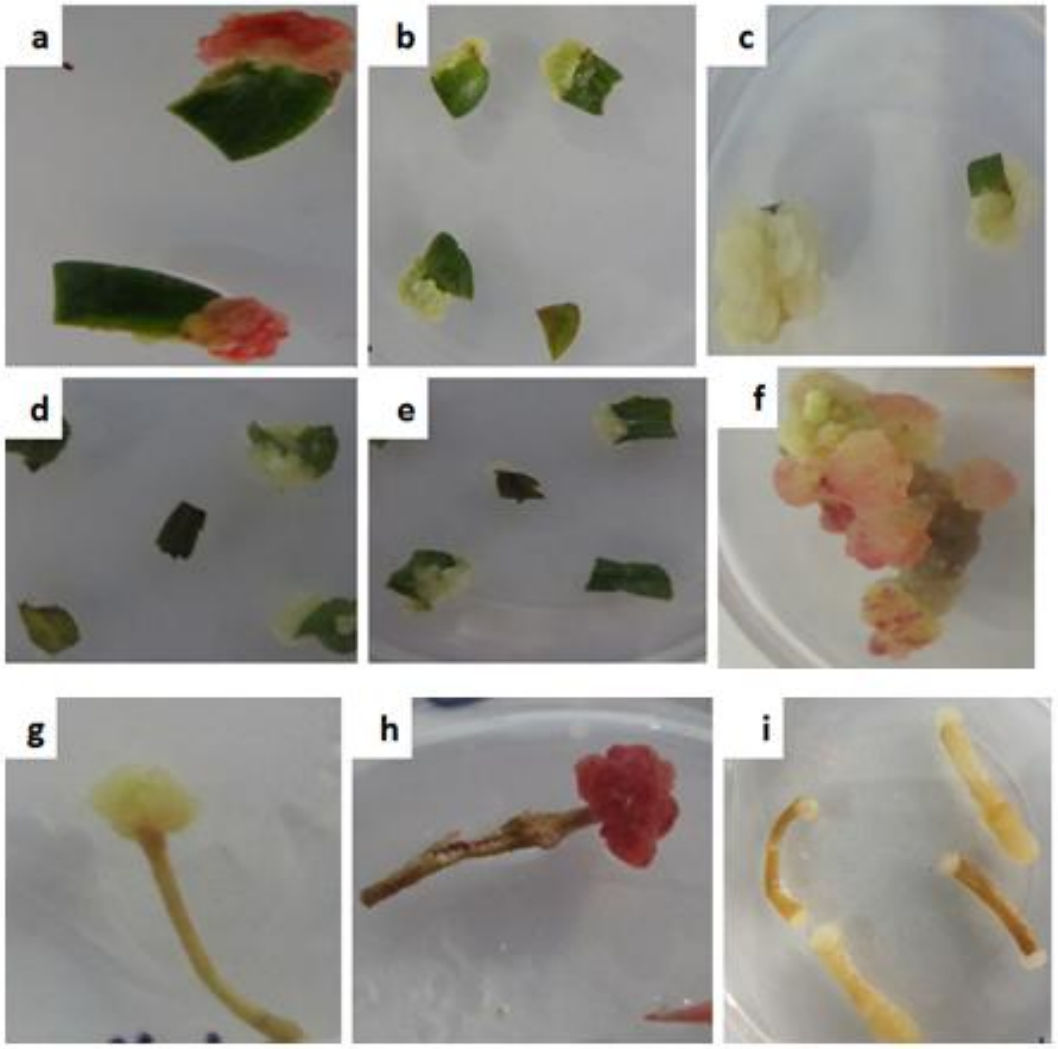

Figure 4.3 : Callus induction from different explants of *C. aronia* on full strength media supplemented with 0.5 mg/L 2,4-D after four weeks under different light condition: (a) callus induced from cotyledon on MS medium under light condition, (b) callus induced from cotyledon on B5 medium under light condition, (c) callus induced from cotyledon on MS medium under dark condition, (d) callus induced from true leaves on MS medium under dark condition, (e) callus induced from true leaves on B5 medium under dark condition, (f) and (h) callus induced from root on B5 medium under light condition, (g) callus induced from root on MS medium under light condition, (i) callus induced from root on B5 medium under dark condition.

4.1.4.2 Effect of Basal Media and Light on Callus Growth

To evaluate the effect of different media type in combination with light and dark on callus growth, callus fresh weight on B5 and MS media each supplemented with 0.5 mg/L 2,4-D under light and dark condition was studied. For callus induced from cotyledon, leaves, and roots callus fresh weight was recorded after four weeks and presented in Figures 4.4, 4.5, 4.6 respectively.

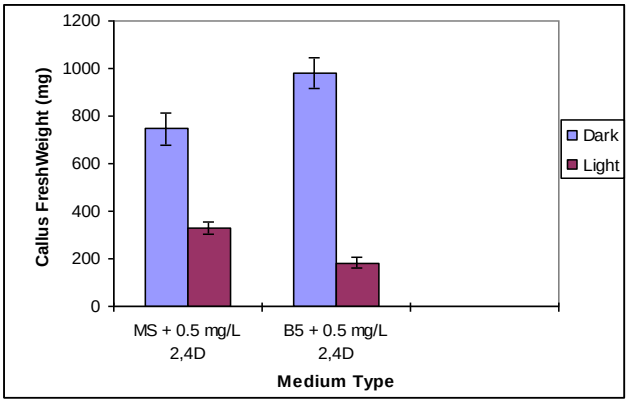

Figure 4.4: The effect of light condition together with different media types on cotyledonary callus growth after four weeks of culture. Columns represent mean callus fresh weight ± SE (standard error). Blue columns represent the dark condition whereas the pink columns represent the light condition. Sample size (n) = 16.

The effect of media type together with light on cotyledonary callus growth was tested. Two-way ANOVA showed that media types, light, and the interaction between media types and light gave statistically significant effect on callus growth ($p < 0.05$). B5 medium supplemented with 0.5 mg/L 2,4-D that was cultured under dark condition gave the highest mean callus fresh weight (981.6 ± 65.0 mg) compared to the other media.

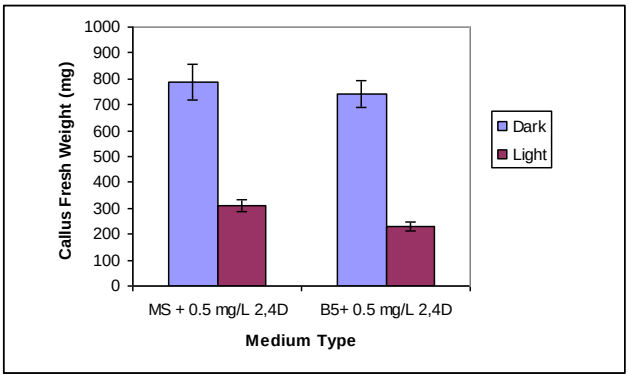

Figure 4.5: The effect of light condition together with different media types on callus-induced from leaves- growth after four weeks of culture. Columns represent mean callus fresh weight ± SE (standard error). Blue columns represent the dark condition whereas the pink columns represent the light condition. Sample size (n) = 16.

The effect of media type and light on callus-induced from leaves- growth was tested. Two-way ANOVA showed that light conditions gave statistically significant effect on callus growth ($p < 0.05$). Media type and the interaction between light condition and media type gave no statistically significance result. MS medium supplemented with 0.5 mg/L 2,4-D that was cultured under dark condition gave the highest mean callus fresh weight (789.0 ± 68.8 mg) compared to the other media.

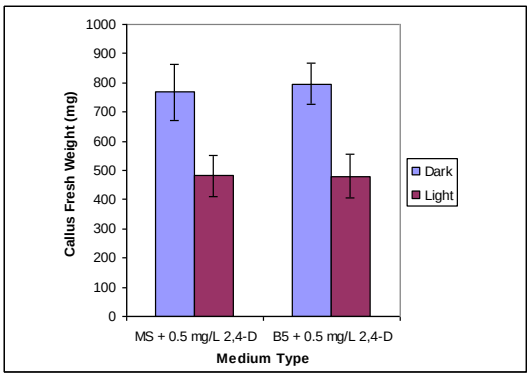

Figure 4.6: The effect of light condition together with different media types on callus-induced from roots- growth after four weeks of culture. Columns represent mean callus fresh weight ± SE (standard error). Blue columns represent the dark condition whereas the pink columns represent the light condition. Sample size (n) = 16.

The effect of media type and light on callus induced from roots growth was tested. Two-way ANOVA showed that light have statistically significant effect on callus growth ($p < 0.05$). Media type and the interaction between light condition and media type gave no statistically significance result. B5 medium supplemented with 0.5 mg/L 2,4-D that was cultured under dark condition gave the highest mean callus fresh weight (796.8 ± 71.5 mg) compared to the other media.

From previous result we can conclude that callus grown under dark condition had higher fresh weight than that under light condition despite the explant type. These differences in callus growth rate might be attributed to the difference in the basal salt formulation and the interaction between the basal media and light condition.

C. aronia callus grown in the dark lost their green pigmentation and showed pale yellow color. Texture of the callus grown in the dark was also different from callus grown in the light. The dark-grown callus is softer and more wet than light-grown callus. B5 medium exhibit friable callus when compared to MS that gave compacted callus specially the callus that was induced from leaves. It is well established in plant tissue culture the effect of light on callus growth. In a study addressed the effect of light on callus induced from three tomato cultivars Jaramillo and Summers (1991) showed that the number of anthers producing callus and callus diameter increased with prolonged periods of dark exposure. They found that calli grown for ten weeks in darkness were 3.4 times larger than calli exposed to five weeks of darkness followed by five weeks of light. Moreover, the study of Summart et al., (2008) showed for callus of Thai aromatic rice seeds variety KDML 105, calli grown under dark condition had higher cell mass than that under light condition. In contrast to the current study results, Artanti and Mcfarlane (1996) found that callus culture of *Coronilla rostrata* had higher fresh weight compared to cultures grown under complete darkness or continuous low light intensity. They reported that increasing the light intensity could increase the callus growth, due to better uptake of some nutrient in the media. Furthermore, Afshari et al. (2011) found that light had a significant effect on increasing *Brassica napus* callus fresh weight both in cotyledon and hypocotyl derived calli compared to callus cultured under dark condition.

4.1.4.3 Estimation of Callus Growth Curve

The callus growth curve was generated by taking the fresh weight of induced callus from three Petri-dishes every week during five to six weeks of culture. The growth curves of callus induced from cotyledons, callus induced from leaves, and callus induced from roots are presented in Figures 4.7, 4.8, and 4.9 respectively.

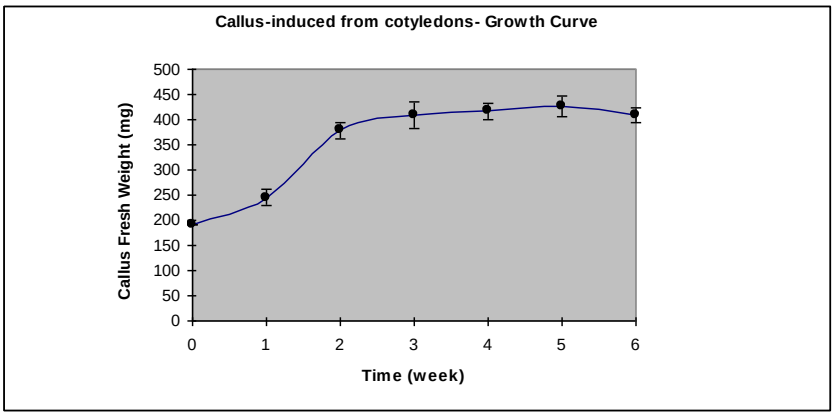

Figure 4.7: Growth curve illustrating change in fresh weight of *C. aronia* callus induced from cotyledons on full strength B5 medium supplemented with 0.5 mg/L 2,4-D under light condition. Initial weight of inoculum was 190 mg (fresh weight). Sample size (n) = 12.

According to the shape of the curve (S shape), the best time for subculturing callus is the time between the 3th and 4th week because the curve attend after that to enter the stationary phase. In the fifth week callus was at the end of lag phase.

To the best of my knowledge, it is the first report where a growth curves of callus induction from cotyledons, leaves, and roots is generated .

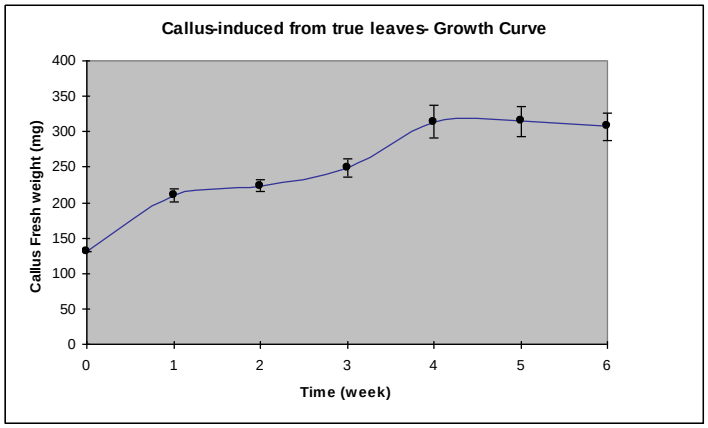

Figure 4.8: Growth curve illustrating change in fresh weight of *C. aronia* callus induced from true leaves on full strength MS medium supplemented with 0.5 mg/L 2,4-D under light condition. Initial weight of inoculum was 130 mg (fresh weight). Sample size (n) = 12.

For callus that induced from leaves the best time for callus subculture is the time between the 4th and 5th week because the curve attend after that to move to the stationary phase.

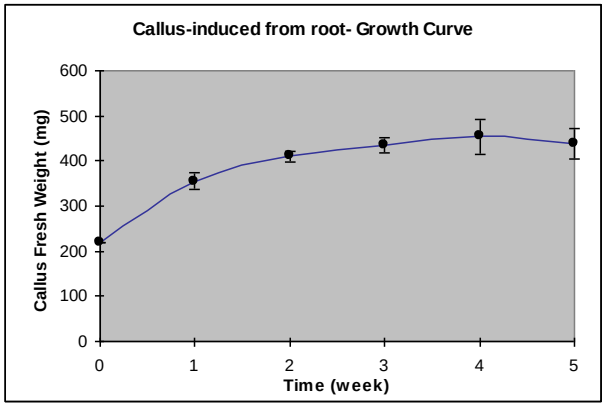

Figure 4.9: Growth curve illustrating change in fresh weight of *C. aronia* callus induced from root on full strength B5 medium supplemented with 0.5 mg/L 2,4-D under light condition. Initial weight of inoculum was 220 mg (fresh weight). Sample size (n) = 12.

For callus that induced from roots the best time for callus subculture is the time between the 3rd and 4th week because the growth attend after that to enter the stationary phase.

4.1.4.4 Callus Growth and Subculture

To find out the optimum medium for callus growth, fresh weight of subcultured callus in eight different media types was taken after four weeks. (B5 for callus induced from cotyledons, and MS for callus induced from leaves). Data were recorded after four weeks and presented in Table 4.8.

Table 4.8: Average fresh callus weight in mg that were cultured on B5, and MS media supplemented with 0.1, 0.5 mg/L NAA and 0.1, 0.5, 1.0 mg/L TDZ and BA for callus induced from cotyledons, and for callus induced from leaves respectively. Sample size (n) = 24.

Regulator Combinations	**Callus Induced from Cotyledons Mean Fresh Weight (mg) ± SE**	**Callus Induced from leaves Mean Fresh Weight (mg) ± SE**
0.1 mg/L TDZ + 0.1 mg/L NAA	328.4 ± 14.4	524.4 ± 23.2
0.5 mg/L TDZ + 0.1 mg/L NAA	422.8 ± 26.8	522.4 ± 22.0
0.5 mg/L TDZ + 0.5 mg/L NAA	623.2 ± 34.8	759.7 ± 27.2
1.0 mg/L TDZ + 0.5 mg/L NAA	708.5 ± 30.7	776.4 ± 35.6
0.1 mg/L BA + 0.1 mg/L NAA	504.1 ± 19.5	342.7 ± 17.7
0.5 mg/L BA + 0.1 mg/L NAA	573.4 ± 23.9	426.9± 34.4
0.5 mg/L BA + 0.5 mg/L NAA	562.2 ± 19.6	548.0 ± 41.6
1.0 mg/L BA + 0.5 mg/L NAA	526.4 ± 24.0	482.9 ± 33.2

The significance between treatments for callus induced from cotyledons was tested by One-Way ANOVA. Results showed significant differences ($p < 0.05$) between the eight tested media pertaining to their effect on callus growth. In the present study, it is concluded that increasing the level of TDZ results with an increase in callus fresh weight. On the other hand increasing level of BA has inhibitory effect on callus fresh weight. B5 medium supplemented with 1.0 mg/L TDZ and 0.5 mg/L NAA gave the highest callus fresh weight. The mean callus fresh weight at this level is 708.5 ± 30.7 mg. Callus that was cultured on B5 with TDZ hormone appeared green in color and compact when compared to B5 media supplemented with BA hormone which was pale green and less compact.

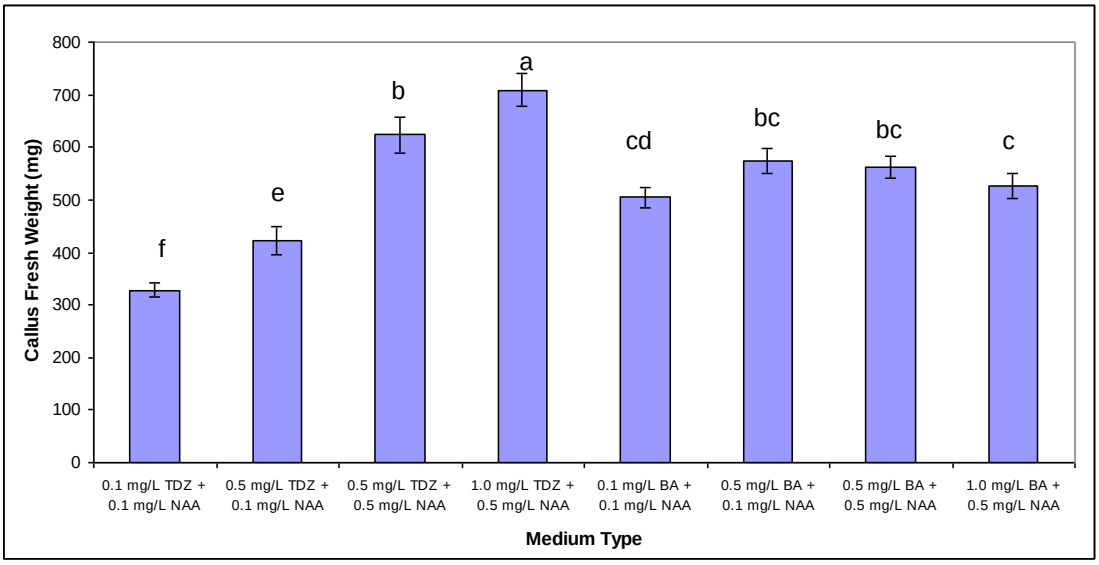

Figure 4.10: The effect of different cytokinins types and concentrations on cotyledonary callus growth after four weeks of culture. Columns represent mean callus fresh weight ± SE (standard error). Different letters accompanying each bar indicate a significant difference by Fisher LSD ($p < 0.05$). Sample size (n) = 24.

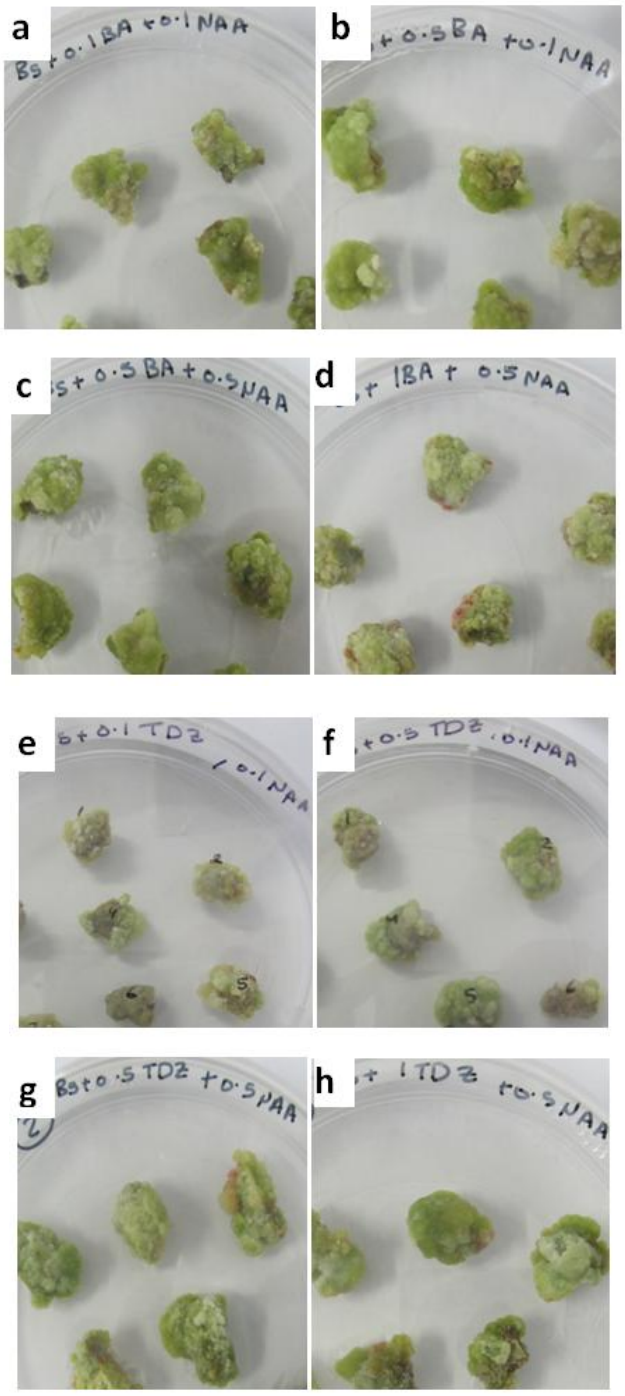

Figure 4.11 : Growth of cotyledonary callus after four weeks of culture on B5 media supplemented with different concentration of BA, TDZ and NAA. (a) B5 media supplemented with 0.1 mg/L BA and 0.1 mg/L NAA. (b) B5 media supplemented with 0.5 mg/L BA and 0.1 mg/L NAA. (c) B5 media supplemented with 0.5 mg/L BA and 0.5 mg/L NAA. (d) B5 media supplemented with 1.0 mg/L BA and 0.5 mg/L NAA. (e) B5 media supplemented with 0.1 mg/L TDZ and 0.1 mg/L NAA. (f) B5 media supplemented with 0.5 mg/L TDZ and 0.1 mg/L NAA. (g) B5 media supplemented with 0.5 mg/L TDZ and 0.5 mg/L NAA. (h) B5 media supplemented with 1.0 mg/L TDZ and 0.5 mg/L NAA.

For callus induced from leaves the significance between treatments was tested by One-Way ANOVA. Results showed significant differences ($p < 0.05$) between the eight tested media pertaining to their effect on callus growth. In the current study, it is concluded that increasing TDZ level slightly increased callus fresh weight, while increasing level of BA has inhibitory effect on callus fresh weight. This result is compatible with Ozden and Karaaslan (2011) result on callus growth of *Vitis vinifera*. They showed that increasing BA concentration inhibit callus growth and induced apoptosis. MS medium supplemented with 1.0 mg/L TDZ and 0.5 mg/L NAA gave the highest callus fresh weight. The mean callus fresh weight at this level is 776.4 ± 35.6 mg. Callus induced from leaves that was cultured on MS with TDZ or BA hormone gave green color with small red pigmented zone in some of callus clumps and callus has compact texture. The high level of cytokinin produces higher number of red pigmented zone. Callus that induced from leaves gave higher fresh weight than callus induces from cotyledons at the same TDZ hormone level. For BA hormone callus that induce from cotyledons gave higher fresh weight than callus induced from leaves.

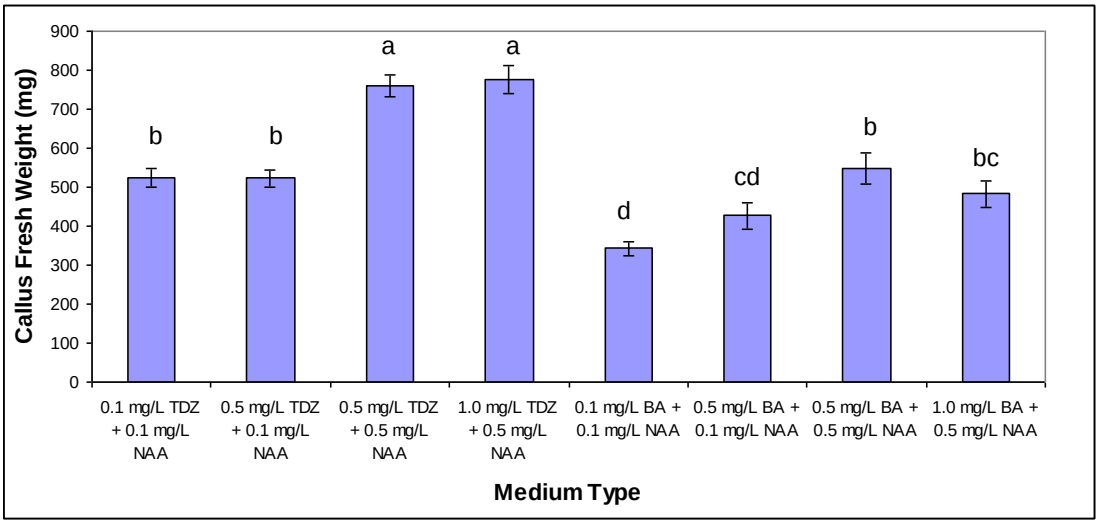

Figure 4.12: The effect of different cytokinins types and concentrations on leaves callus growth after four weeks of culture. Columns represent mean callus fresh weight ± SE (standard error). Different letters accompanying each bar indicate a significant difference by Fisher LSD ($p < 0.05$). Sample size (n) = 24.

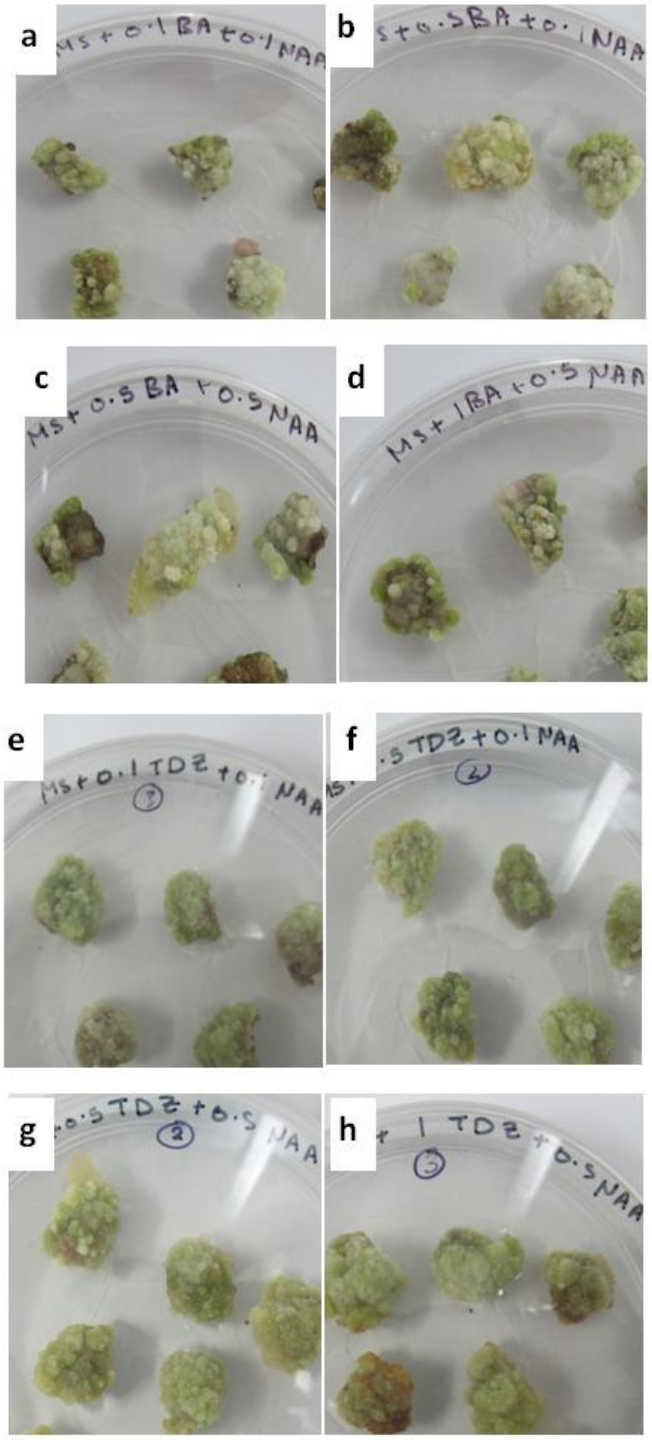

Figure 4.13: Growth of leaves callus after four weeks of culture on MS media supplemented with different concentration of BA, TDZ and NAA. (a) MS media supplemented with 0.1 mg/L BA and 0.1 mg/L NAA. (b) MS media supplemented with 0.5 mg/L BA and 0.1 mg/L NAA. (c) MS media supplemented with 0.5 mg/L BA and 0.5 mg/L NAA. (d) MS media supplemented with 1.0 mg/L BA and 0.5 mg/L NAA. (e) MS media supplemented with 0.1 mg/L TDZ and 0.1 mg/L NAA. (f) MS media supplemented with 0.5 mg/L TDZ and 0.1 mg/L NAA. (g) MS media supplemented with 0.5 mg/L TDZ and 0.5 mg/L NAA. (h) MS media supplemented with 1.0 mg/L TDZ and 0.5 mg/L NAA.

4.1.4.5 Effect of Basal Media on Red colored Callus

To find out the optimum medium for red colored callus growth and maintenance of red pigment, the fresh weight and color of subcultured callus in three different types of media were taken after four weeks. Callus fresh weight was reported in Table 4.9.

Table 4.9: Average fresh weight in mg and color of red colored callus that were cultured on B5, and MS media supplemented with 2.0 mg/L 2,4-D and 1.5 mg/L kinetin after four weeks of culture. Sample size (n) = 24.

Medium type	Mean Callus Fresh Weight (mg) ± SE	Callus Color
B5 + 0.5 mg/L 2,4-D (induction media)	2401.4 ± 104.1	Pink to red
B5 + 2.0 mg/L 2,4-D +1.5 mg/L kinetin	2347.2 ± 75.9	Brown
MS + 2.0 mg/L 2,4-D +1.5 mg/L kinetin	2422.9 ± 144.0	Brown

The significance between treatments was tested by One-Way ANOVA. Results showed no significant differences ($p > 0.05$) between the three tested media pertaining to their effect on callus growth. MS media supplemented with 2.0 mg/L 2,4-D and 1.5 mg/L kinetin gave the highest callus fresh weight (2422.9 ± 144.0 mg) but the red color changed to brown after four weeks.

In the current study, it is found that callus have accumulated colored pigment and appeared in pink to red color. Red callus coloration indicated the presence of high level of polyphenols. Red colored callus was extracted using anthocyanin extraction solution using method reported by Maharik et al., (2009b). The formation of reddish color extract indicating the presence of flavonoids and based on Maharik et al., (2009b) red color extract indicate the presence of anthocyanin in callus culture of *C. aronia*. Red pigment was appeared on callus induction media which is B5 media supplemented with 0.5 mg/L 2,4-D after six months of successive subculture.

Anthocyanins are member of flavonoides occuring almost in all tissue of higher plants exhibiting colors from pale pink to bright red to deep blue pigmentation. Production of anthocyanin in plant cell and tissue cultures has been reported for many species including carrot (*Daucus carota*) (Narayan and Venkataraman, 2002; Sudaha and Ravishankar, 2003), and catharanthus (Taha et al., 2008). Moreover anthocyanin pigmentation was stimulated in callus of *C. sinaica* (Maharik et al., 2009b). It is well known that phenolic compounds are major constituents in hawthorn trees and anthocyanin is found in bark, fruits, and roots of the plant. Thus it is not surprising to

be induced when callus is subjected to anthocyanin inducing condition. In contrast to our result regarding red colored induction media Maharik et al., (2009) reported that pale pink pigmentation of anthocyanin appeared in leaf and stem callus cultures of *C. sinaica* on MS media supplemented with 1.0 mg/L kinetin and 0.5 mg/L NAA after 5 weeks of culture. In general agreement with our result, Al Abdallat et al., (2011) show that deep red colored callus of *C. aronia* that initiated from internodal stem segments was obtained on MS media supplemented with different levels of 2,4-D hormone (0.5, 1, and 2 mg/L). Also callus produced on MS media supplemented with BA gave light red color and in combination treatments with 2,4-D and kinetin produce red color, while 2,4-D and BA gave lighter red coloration. Repeated subcultures of red colored callus decreased the ability of callus cells to produce anthocyanin until it was diminished and the callus changed to brown color and this result supported by Maharik et al., (2009b). In the current study red colored callus cultured on other media in addition to the induction media to obtain the best media in terms of higher callus fresh weight with predominant red color. Our result indicate that the induction media (B5 + 0.5 mg/L 2,4-D) maintained the red color for longer period, however the callus color became more brownish than reddish after 40 days. The present result is in compatible with Al Abdallat et al., (2011) result on *C. aronia* internodal stem segment derived callus. They reported that MS medium supplemented with 2.0 mg/L 2,4-D and 1.5 mg/L kinetin produced higher average callus fresh weight when compared to the induction on MS media supplemented with 5.0 mg/L IBA and 0.5 mg/L BA. Callus that was cultured on MS medium supplemented with 2.0 mg/L 2,4-D and 1.5 mg/L kinetin have a predominant red coloration indicating the presence of high levels of polyphenols. In the current study it has been found that neither B5 nor MS media supplemented with 2.0 mg/L 2,4-D and 1.5 mg/L kinetin enhanced the anthocyanin production in cotyledonary callus of *C. aronia* and the red color turned to brown. Furthermore, Maharik et al., (2009b) reported that different combination of kinetin and NAA did not increase anthocyanin production. However, substitution of kinetin by 1.0 mg/L BA in culture medium combined with 0.5 mg/L NAA increased anthocyanin accumulation and restored anthocyanin production through repeated subcultures. This result supports our result on callus induced from leaves in which MS media supplemented with the previous hormone combination produce red pigmented zones in callus clumps which indicate the stimulation of anthocyanin in leaves derived callus. These reports showing the different growth

regulators which stimulates anthocyanin production in different *Crataegus* species. This attributed to the variability of anthocyanin stimulation requirements across different plant species in this genus.

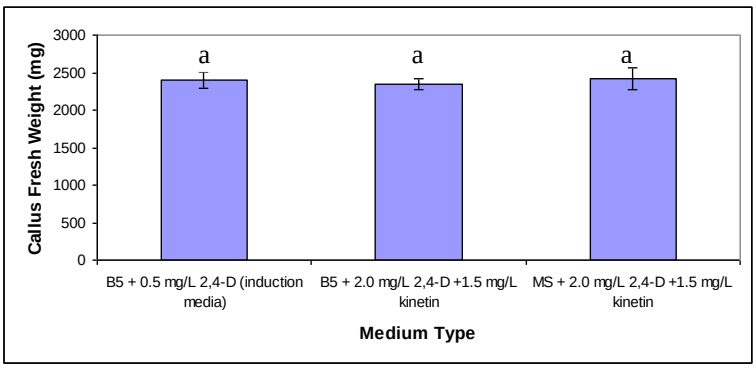

Figure 4.14: The effect of different media types on red colored callus growth after four weeks of culture. Columns represent mean of callus fresh weight ± SE (standard error). Different letters accompanying each bar indicate a significant difference by Fisher LSD ($p < 0.05$). Sample size (n) = 24.

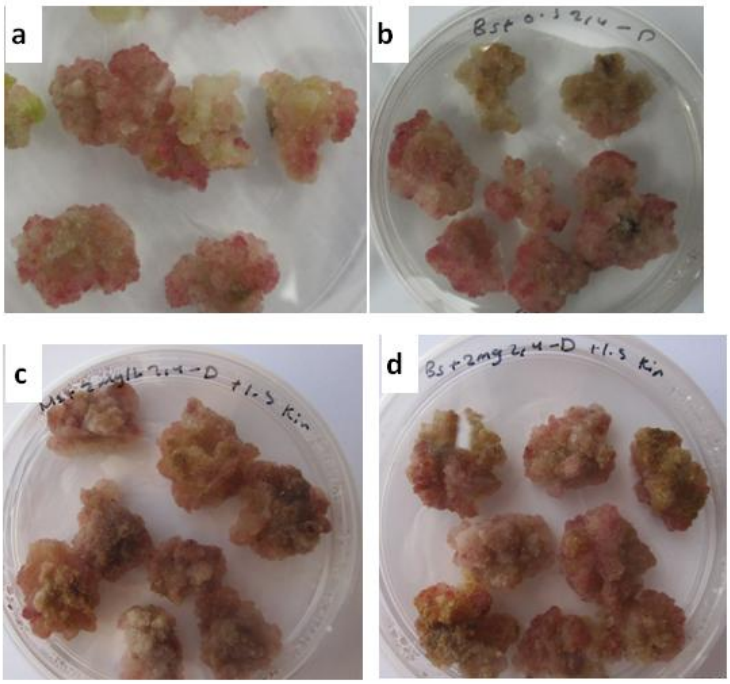

Figure 4.15: Red colored callus growth and color after four weeks of culture on different media. (a) initiations red colored callus on B5 media supplemented with 0.5 mg/L 2,4-D. (b) red colored callus after four weeks with increase of phenolic compound which appear as brown color. (c) red colored callus after four weeks of culture on MS media supplemented with 2 mg/L 2,4-D and 1.5 mg/L kinetin. (d)) red colored callus after four weeks of culture on B5 media supplemented with 2 mg/L 2,4-D and 1.5 mg/L kinetin.

4.1.4.6 Effect of Media with Antioxidant on Cotyledonary Callus and Red Colored Callus Growth

To minimize browning in callus induced from cotyledons and to maintain red pigment of red colored callus and to prevent it from decomposition, four antioxidant treatments were carried out based on addition of charcoal, PVP, citric acid, and ascorbic acid. After four weeks of callus culture on B5 media supplemented with 0.5 mg/L 2,4-D and one of the mention antioxidant, callus fresh weight was taken and represented in Figure 4.16.

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 three tested treatments pertaining to their effect on callus growth. B5 media supplemented with 0.5 mg/L 2,4-D and 1.0 g/L PVP resulted with the highest callus fresh weight and qualitatively the least in phenolic browning.

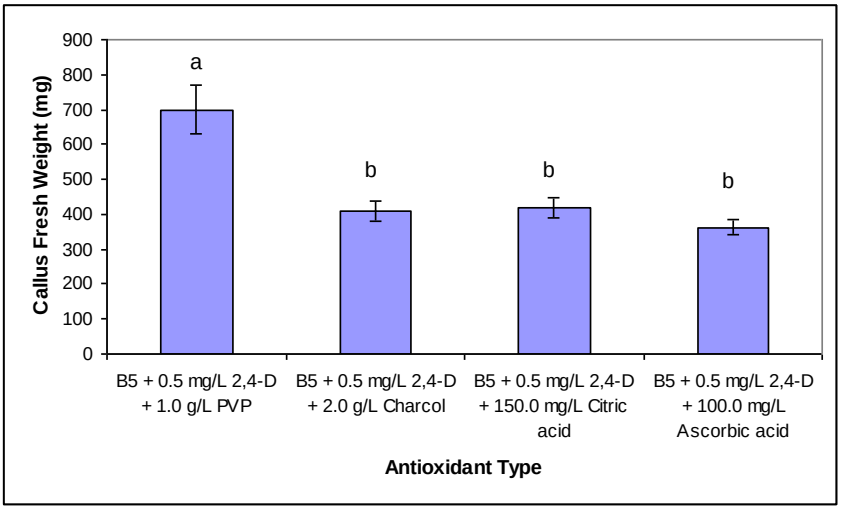

Figure 4.16: Growth response to different antioxidant treatments on cotyledonary callus after four weeks of culture. Columns represent mean callus fresh weight ± SE (standard error). Different letters accompanying each bar indicate a significant difference by Fisher LSD ($p < 0.05$). Sample size (n) = 16.

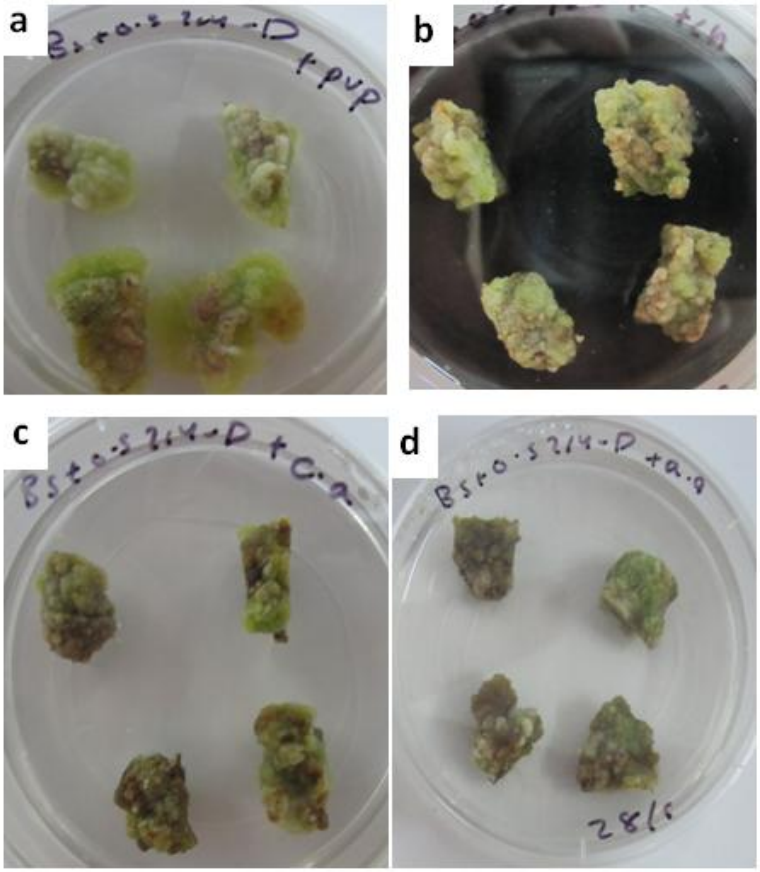

Figure 4.17: Growth and color of cotyledonary callus after four weeks of culture on B5 media supplemented with 0.5 mg/L 2,4-D and different antioxidant treatments. (a) 1g/L PVP, (b) 2 g/L charcoal, (c) 150 mg/L citric acid, (d) 100 mg/L ascorbic acid.

Red colored callus were cultured on B5 media supplemented with 0.5 mg/L 2,4-D and one of the previously mentioned antioxidant. Callus fresh weight in response to different antioxidant treatment were taken after four weeks and represented in Figure 4.18.

The significance between treatments was tested with the analysis of variance by One-Way ANOVA, and the results showed significant differences ($p < 0.05$) between the four tested treatment pertaining to their effect on callus growth. B5 media supplemented with 0.5 mg/L 2,4-D and 1.0 g/L PVP resulted with the highest callus fresh weight and maintenance of the red pigment.

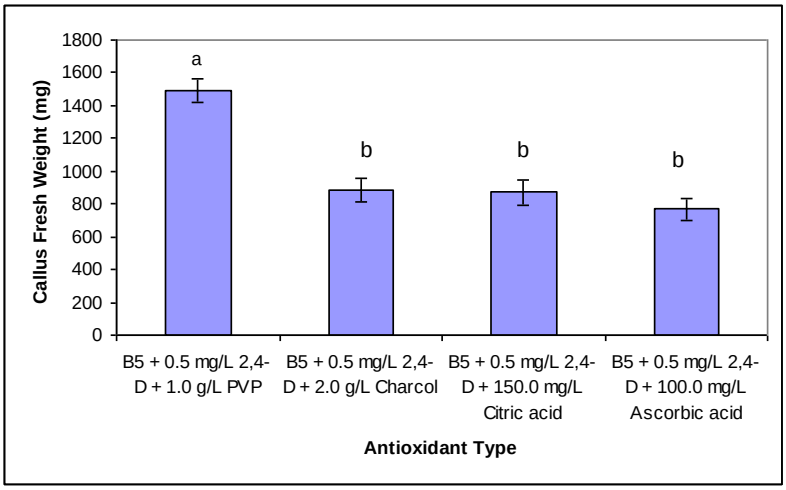

Figure 4.18: Growth response to different antioxidant treatments on red colored callus after four weeks of culture. Columns represent mean callus fresh weight ± SE (standard error). Different letters accompanying each bar indicate a significant difference by Fisher LSD ($p < 0.05$). Sample size (n) = 16.

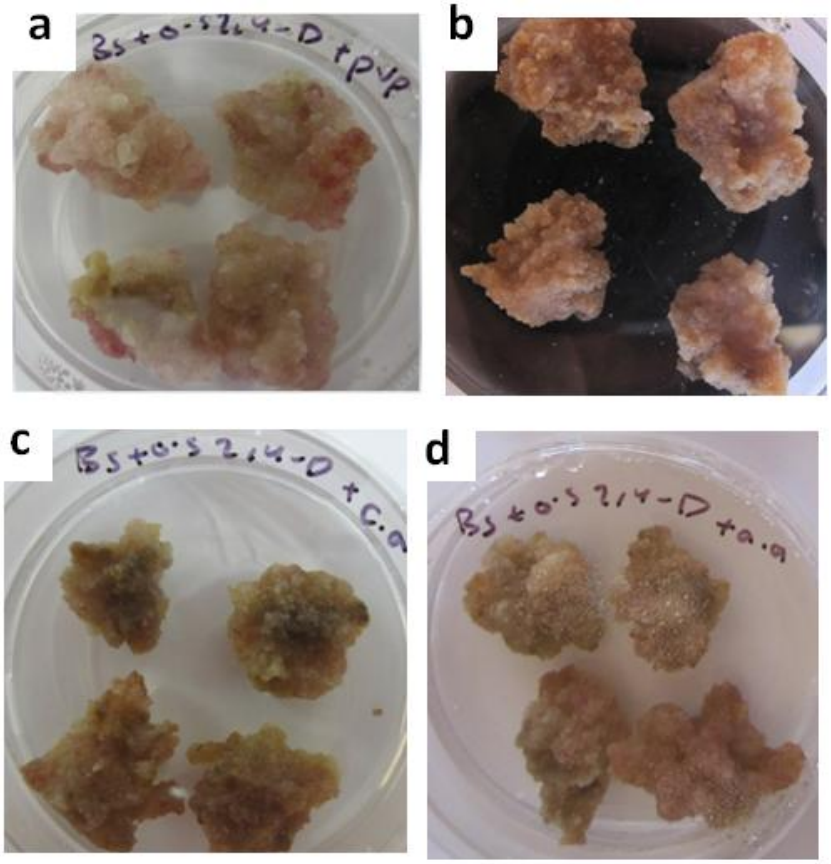

Figure 4.19: Red colored callus growth and color after four weeks of culture on B5 media supplemented with 0.5 mg/L 2,4-D and different antioxidant treatments. (a) 1g/L PVP, (b) 2 g/L charcoal, (c) 150 mg/L citric acid, (d) 100 mg/L ascorbic acid.

In the current study, four antioxidant treatments were carried out in order to minimize phenolics which appeared as browning and necrosis in cotyledon derived callus and in red colored callus. Results show that PVP treatment gave the best antioxidant activity in terms of callus fresh weight and color. Red colored callus clumps that were cultured on media containing PVP are the least callus show browning and maintaining the red pigment followed by citric acid, ascorbic acid and finally charcoal. For cotyledonary callus PVP containing media is also the best media that reduce browning in callus clumps followed by, charcoal, citric acid and finally ascorbic acid.

More attention has been paid to minimize the browning 'phenolization' of plant cultures *in vitro*, commonly by adding antioxidants, such as ascorbic acid or glutathione (GSH), or phenol absorbing polymers like PVP (polyvinylpyrrolidone). The excessive production of polyphenols results from unfavorable or suboptimal culture condition. At the onset of senescence, the decrease in free phenolics and a simultaneous increase in cell-wall bound and oxidized forms is observed in callus culture (Arnalods et al., 2001 cited in Matkowski, 2008). Abdelwahd et al. (2008) reported that PVP was used to treat faba bean seeds, in which seeds were treated with PVP solution (1000 mg/L) for one hour followed by culturing in MS medium supplemented with ascorbic acid (1.0 mg/L) or activated charcoal (10 g/L), greatly reduced lethal browning in explants and improved shoot regeneration. Furthermore, Al Abdallat et al. (2011) showed that callus induction media contained 100 mg/L citric acid as antioxidant. Moreover, Afshari et al. (2011) found that although callus fresh weight of rapeseed (*Brassica napus* L.) have increased in the light, but browning and necrosis of callus increased. This browning attributed to the poly-phenolics compounds present in the plants. These compounds will be oxidized when the explants are cut by the enzyme polyphenoloxidase and this is one of the main reasons of browning in the plant callus (Afshari et al., 2011). It is probably that the products of this oxidation process are formed in light (Afshari et al., 2011), and this explains why browning occurs less in dark. In general agreement with the current study results, Afshari et al., (2011) recommended the addition of antioxidants like ascorbic acid or citric acid to the media, soaking in these antioxidants before culturing in the media or subculture to a fresh media immediately after browning has been observed.

4.2 Extraction of Secondary Metabolites

4.2.1 Extraction Yields

Powdered plant materials of 3.0 g dry weight from each of the following plants: leaves from *ex vitro* growing plants, leaves from *in vitro* plants, callus induced from leaves and callus induced from cotyledons, were soaked in 100 ml acetone/water (70:30) (v/v). Mixtures were stirred for 96 hours at room temperature and supernatant was removed by centrifugation, and solvents were air dried in the fume hood. The percentages of extraction yield were calculated as a result of dividing the weight of the extracted powder by the weight of the extracted plant material.

Table 4.10: Percentage extract yield (%) of 3.0 g of *C. aronia* (*ex vitro* leaves, *in vitro* leaves, and callus) extracted by acetone/water (70/30) (v/v).

Extracted Plant Material	**Extraction Yield (%)**
Ex vitro leaves	27.42
In vitro leaves	33.91
Callus induced from cotyledons	27.31
Callus induced from true leaves	38.07

In this study extraction yields are ranged between 38.07% and 27.31%. According to the results, extract of leaves derived callus gave the highest extraction yield with 38.07%. Extracts of *in vitro* leaves gave higher extraction yield compared to *Ex vitro* leaves.

The dry callus was obtained by drying fresh callus in the oven for 24 hours at 40°C. Fresh and dry callus were weighted and the water amount in callus was calculated. Fresh callus weight was 9.95 g and dry callus weight was 1.01 g. The weight of water in callus was 8.94 g which is correspond to 89.8%.

4.2.2 TLC Analysis and Phenolic Compounds Assay

Extracts derived from four tissues were investigated by TLC method using acetone/water (70:30) (v/v) as solvent and chloroform:acetone:acetic acid at (60:18.5:21.5) as the mobile phase. Our focus was mostly on phenolic compounds which are UV active and showed positive staining with $FeCl_3$ solution. Equal amount of each extract was loaded on TLC plate (1.0µL), run in appropriate solvent and phenolic compound was detected under UV light, and stained with $FeCl_3$ solution,

dark spot was obtained after slow heating. $FeCl_3$ provided an excellent mean of selectively visualizing phenols. R*f* value of the major compound was 0.314.

According to TLC image we most probably have one compound (spot in the middle indicated by the arrow). Also TLC image reflects different concentrations and enrichment of the compound in the *in vitro* and callus material. The major phenolic compound was isolated from *in vitro* leaves extract using plate chromatography and finally investigated by mass spectroscopy for molecular weight determination.

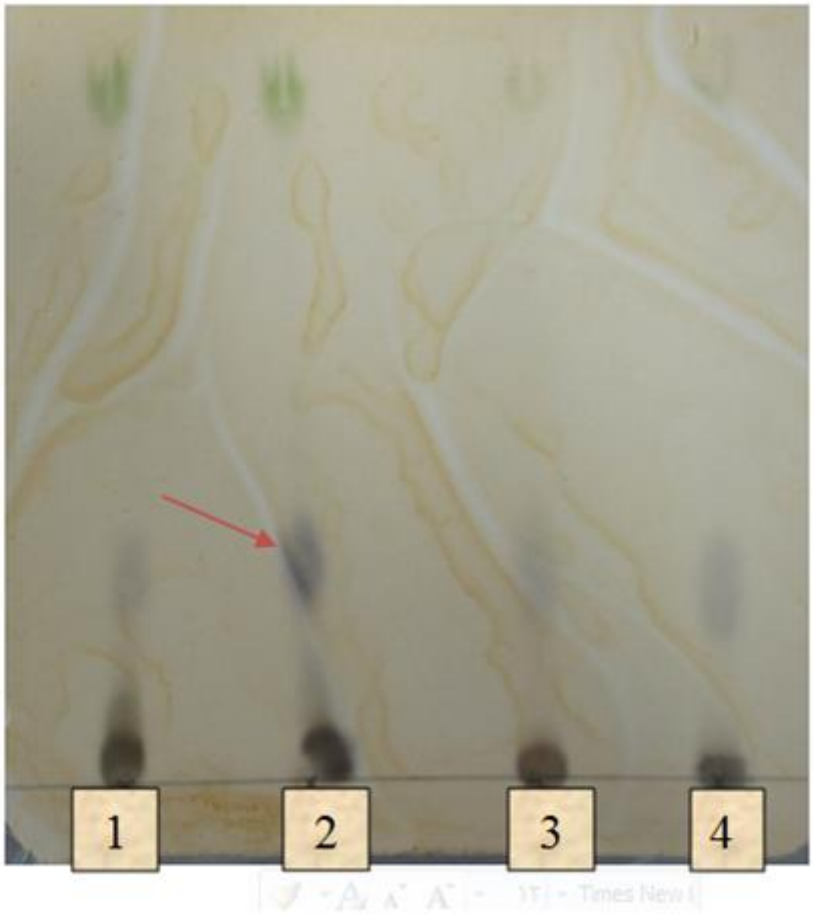

Figure 4.20: TLC plate spotted with acetone/water (70:30) (v/v) extract from different tissues of *C. aronia* after being sprayed with $FeCl_3$. (1) *ex vitro* leaves of *C. aronia*, (2) *in vitro* leaves of *C. aronia*, (3) callus induced from true leaves of *C. aronia*, (4) callus induced from cotyledons of *C. aronia*.

4.2.3 Separation and Characterization of the Major Compound

Mass spectroscopy picture gives major peak indicated by the red arrow. The peak corresponds to phenolic compound of molecular weight 353.31g/mole which is similar to chlorogenic acid. Other peaks were corresponding to impurities from the surface of plastic material which is most probably from eppendorf tube. These impurities are frequently appeared in samples with very low quantity. The accumulation of chlorogenic acid in callus culture of *Crataegus* have been reported in literature; Bahorun et al. (1994) confirmed the accumulation of chlorogenic acid in callus culture of *Crataegus monogyna* besides proanthocyanin, epicatechin, and many other phenolic compound. Moreover Zhang et al. (2001) showed that hawthorn (*Crataegus pinnatifida*) fruits contain active antioxidants of the phenolic type. The column chromatographic separation led to isolation of seven antioxidants (specifically, hyperoside, isoquercitrin, epicatechin, chlorogenic acid, quercetin, rutin, and protocatechuic acid).

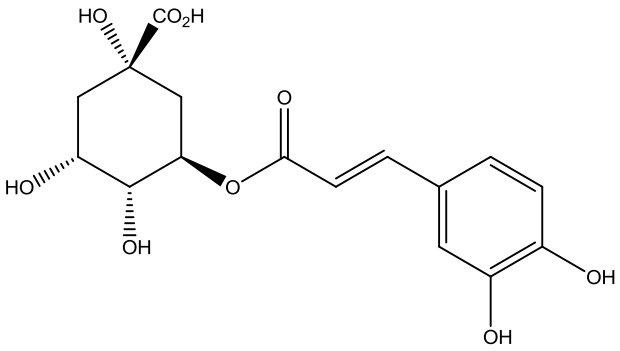

Figure 4.21: Chlorogenic acid structure

The antioxidant and biological effects of chlorogenic acid and its analogues have been described. Zhang et al. (2001) reported that the chlorogenic acid and its analogues protected low density lipoprotein (LDL) and α-tocopherol from oxidation and increased the level of serum α-tocopherol in rats fed a 2% hawthorn fruit diet when compared with control. Another study conducted by Chang et al. (2001) determined the presence of four active polyphenol components of Chinese hawthorn, chlorogenic acid, epicatechin, hyproside and isoquercitrin, in rat plasma as biological fluids after consumption of hawthorn. Moreover, Hosseinimehr et al. (2007) showed that *Crataegus microphylla* fruit extract exhibited concentration-dependent activity on 1,1-diphenyl 2-picrylhydrazyl free radical showing that *C. microphylla* rich in

phenolic compounds. HPLC analysis showed that it contained chlorogenic acid, epicatechin and hyperoside. They reported that *C. microphylla* extract with antioxidant activity reduced the genotoxicity induced by gamma irradiation in bone marrow cells. Furthermore, Sahloul et al. (2009) reported that hawthorn (*Crataegus azarolus*) from Tunisia contains eight antioxidant of phenolic type: chlorogenic acid, hyperoside, rutin, spiraeoside, isoquercitrin, quercetin, epicatechin and the dimer procyanidin B2. These compound identified specially in extracts of floral buds and presented a strong radical-scavenging activity. Another study conducted by Liu et al. (2010a) characterized phenolics in the crude and the polyamide column chromatography of the ethanolic extract of Chinese hawthorn fruit (*C. pinnatifida*). Forty-two phenolic compounds were detected including chlorogenic acid as one of them. The previous studies support the presence of chlorogenic acid in extract of *in vitro* culture or *ex vitro* tissues of different hawthorn species. These studies support our result of accumulation of chlorogenic acid in callus and *in vitro* culture of *C. aronia*.

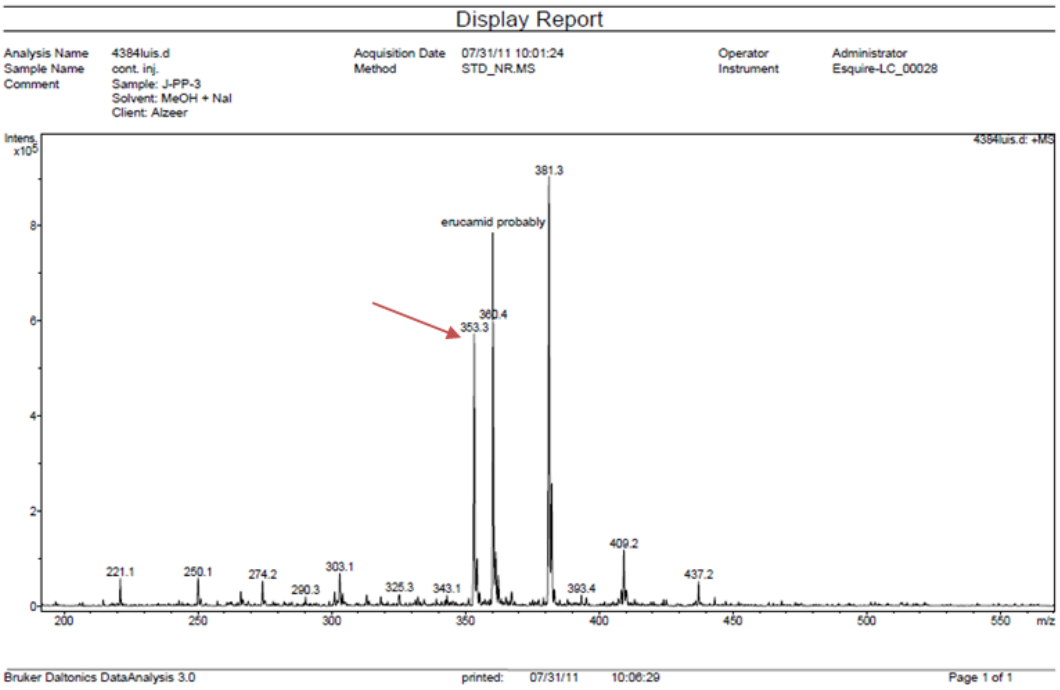

Figure 4.22: Mass spectrum of the phenolic compound from the extract of *in vitro* leaves of *C. aronia*.

CHAPTER 5

CONCLUSION AND FUTURE WORK

5.1 Conclusion

In this study it can be concluded that there was necessity to apply *in vitro* propagation of *C. aronia*, for difficultness and unsuccessful propagation through conventional methods and the risk of losing genetic diversity of this plant. In the current study, seeds are considered good starting material for *in vitro* establishment of *C. aronia* due to high percentage of *in vitro* germination which reached to 100%. QL media is recommended for shoot proliferation since it gives the highest number of shoots when compared to MS or Mcc media. For shoot proliferation QL media supplemented with 2.0 mg/L zeatin and 0.05 mg/L IAA was the appropriate media since it gives the highest number of shoots comparing to BA and kinetin hormone. Roots was failed to be induced at (0.5, 1.0, 1.5 mg/L) of IAA, IBA, and NAA PGR. In the current study, callus was successfully induced from different explants including, cotyledons, true leaves and roots on MS or B5 media supplemented with 0.5 mg/L 2,4-D under light or dark condition. Callus growth under dark condition gave the highest fresh weight despite explants type. Additionally, B5 media supplemented with 1.0 mg/L TDZ and 0.5 mg/L NAA was recommended for maintenance of cotyledonary callus. While for maintenance of leaves derived callus MS media supplemented with 1.0 mg/L TDZ and 0.5 mg/L NAA was recommended. B5 media supplemented with 0.5 mg/L 2,4-D is the suitable for red pigment induction and PVP is the best antioxidant treatment in terms of callus fresh weight and decreasing browning 'phenolization' of red colored callus and cotyledon derived callus. For TLC analysis chloroform:acetone:acetic acid at (60:18.5:21.5) was the appropriate TLC solvent system to detect major phenolic compound. According to TLC result we have enrichment of the compound in *in vitro* leaves and callus. For MS result we have a phenolic compound of 353.3 g/mole molecular weight which may correspond to chlorogenic acid.

5.2 Future Work

Based to the tissue culture part, *in vitro* rooting needs further study in depth of other root induction stimuli to reach the perfect last step for saving our valuable native hawthorn. Further study is needed to maintain and increase the red pigment and find the subculturing stage at which callus accumulates the maximum amount of it. Chromatographic analysis such as HPLC must be used to characterize the red pigment and confirm the presence of chlorogenic acid or any other compounds. The produced powder extracts should be subjected to bioassay analysis concerning anti-cancer activity.

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APPENDICES

- Appendix Table 1: Analysis of variance (ANOVA) for number of leaves at different media type after four weeks of *in vitro* multiplication of mother stock of *C. aronia* in test tubes.

Summary				
Groups	Sample size	Sum	Mean	Variance
QL+ 0.5 Z	10	96	9.6	1072
Mcc+ 0.5 Z	10	117	11.7	1431
MS + 0.5 Z	10	114	11.4	1364
Total	30		10.9	10.43793

ANOVA							
Source of Variation	d.f.	SS	MS	F	p-level	F crit	Omega Sqr.
Between Groups	2	25.8	12.9	1.257855	0.300395	3.354131	0.0169
Within Groups	27	276.9	10.25556				
Total	29	302.7					

Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	-2.1	1.466306	0.153325	rejected
1 vs 3	-1.8	1.256834	0.218843	rejected
2 vs 3	0.3	0.209472	0.835544	rejected

- Appendix Table 2: Analysis of variance (ANOVA) for number of shoots at different media type after four weeks of *in vitro* multiplication of mother stock of *C. aronia* in test tubes.

Summary				
Groups	Sample size	Sum	Mean	Variance
QL+ 0.5 Z	10	10	1	0
Mcc+ 0.5 Z	10	10	1	0
MS + 0.5 Z	10	10	1	0

ANOVA						
Source of Variation	SS	df	MS	F	p-level	F crit
Between Groups	0	2	0	#N/A	#N/A	#N/A
Within Groups	0	27	0			
Total	0	29				

- Appendix Table 3: Analysis of variance (ANOVA) for shoot height at different media type after four weeks of *in vitro* multiplication of mother stock of *C. aronia* in test tubes.

Summary				
Groups	Sample size	Sum	Mean	Variance
QL+ 0.5 Z	10	9.5	0.95	10.79
Mcc+ 0.5 Z	10	15.3	1.53	25.97
MS + 0.5 Z	10	12.5	1.25	18.57
Total	30		1.243333	0.308747

ANOVA							
Source of Variation	d.f.	SS	MS	F	p-level	F crit	Omega Sqr.
Between Groups	2	1.682667	0.841333	3.124192	0.060188	3.354131	0.124046
Within Groups	27	7.271	0.269296				
Total	29	8.953667					

Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	-0.58	2.499182	0.01836	accepted
1 vs 3	-0.3	1.29268	0.206327	rejected
2 vs 3	0.28	1.206501	0.237375	rejected

- Appendix Table 4: Analysis of variance (ANOVA) for number of leaves at different media type after six weeks of shoot multiplication of mother stock of *C. aronia* in flasks**.**

Summary				
Groups	Sample size	Sum	Mean	Variance
MS +0.2 kin + 0.05 IAA	12	149	12. 25	1931
Mcc + 0.2 kin +0.05 IAA	12	141	11.75	1785
QL + 0.2 kin +0.05 IAA	12	147	12. 41667	1909
Total	36		12.13889	9.151587

ANOVA							
Source of Variation	d.f.	SS	MS	F	p-level	F crit	Omega Sqr.
Between Groups	2	2.88889	1.444444	0.150171	0.861146	3.284918	-0.04955
Within Groups	33	317.417	9.618687				
Total	35	320.306					

Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	0.666667	0.526534	0.601838	rejected
1 vs 3	0.166667	0.131634	0.896028	rejected
2 vs 3	-0.5	0.394901	0.695313	rejected

- Appendix Table 5: Analysis of variance (Kruskal-Wallis ANOVA) for number of shoots at different media type after six weeks of shoot multiplication of mother stock of *C. aronia* in flasks.

Comparing Multiple Independent Samples			
Sample size	Sum of Ranks		
MS +0.2 kin + 0.05 IAA	12	192	
Mcc + 0.2 kin +0.05 IAA	12	209.5	
QL + 0.2 kin +0.05 IAA	12	264.5	
H	2.149024	N	36
Degrees Of Freedom	2	p-level	0.341464
H (corrected)	5.963542		
Median Test			
Overall Median	1	Chi-square	21.66667
p-level	0.0000		

- Appendix Table 6: Analysis of variance (ANOVA) for shoot height at different media type after six weeks of shoot multiplication of mother stock of *C. aronia* in flasks.

Summary				
Groups	Sample size	Sum	Mean	Variance
MS +0.2 kin + 0.05 IAA	12	13.9	1.158333	18.11
lnMcc + 0.2 kin +0.05 IAA	12	-0.91	-0.07583	1.8213
arsinQL + 0.2 kin +0.05 IAA	12	9.73	0.810833	10.5007
Total	36		0.631111	0.459804

ANOVA							
Source of Variation	d.f.	SS	MS	F	p-level	F crit	Omega Sqr.
Between Groups	2	9.720406	4.860203	25.16758	0	3.284918	0.573132
Within Groups	33	6.37275	0.193114				
Total	35	16.09316					

Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	1.234167	6.879279689	0	accepted
1 vs 3	0.3475	1.936974767	0.060853	rejected
2 vs 3	-0.88667	4.942304922	0	accepted

- Appendix Table 7: Analysis of variance (Kruskal-Wallis ANOVA) for number of leaves at different cytokinins level after six weeks of shoot multiplication of mother stock of *C. aronia* in flasks.

Comparing Multiple Independent Samples			
Sample size	Sum of Ranks		
QL + 0.2 kin +0.05 IAA	12	1033.5	
QL + 1 kin +0.05 IAA	12	549	
QL + 2 kin +0.05 IAA	12	498.5	
QL + 0.5 BA +0.05 IAA	12	585.5	
QL + 1 BA +0.05 IAA	12	649.5	
QL + 2 BA +0.05 IAA	12	649	
QL + 0.5 Z +0.05 IAA	12	742	
QL + 1 Z +0.05 IAA	12	846.5	
QL + 2 Z +0.05 IAA	12	974.5	
Ql control	12	732	
H	19.6782	N	120
Degrees Of Freedom	9	p-level	0.020006
H (corrected)	19.97507		
Median Test			
Overall Median	8.5	Chi-square	23.33333
p-level	0.00549		

- Appendix Table 8: Analysis of variance (Kruskal-Wallis ANOVA) for number of shoots at different cytokinins level after six weeks of shoot multiplication of mother stock of *C. aronia* in flasks.

Comparing Multiple Independent Samples			
Sample size	Sum of Ranks		
QL + 0.2 kin +0.05 IAA	12	809	
QL + 1 kin +0.05 IAA	12	631.5	
QL + 2 kin +0.05 IAA	12	631.5	
QL + 0.5 BA +0.05 IAA	12	576	
QL + 1 BA +0.05 IAA	12	702	
QL + 2 BA +0.05 IAA	12	835	
QL + 0.5 Z +0.05 IAA	12	698	
QL + 1 Z +0.05 IAA	12	809	
QL + 2 Z +0.05 IAA	12	936.5	
QL control	12	631.5	
H	8.307163	N	120
Degrees Of Freedom	9	p-level	0.503513
H (corrected)	16.56899		
Median Test			
Overall Median	1	Chi-square	51.66667
p-level	0		

- Appendix Table 9: Analysis of variance (Kruskal-Wallis ANOVA) for shoot height at different cytokinins level after six weeks of shoot multiplication of mother stock of *C. aronia* in flasks.

Comparing Multiple Independent Samples			
Sample size	Sum of Ranks		
QL + 0.2 kin +0.05 IAA	12	859.5	
QL + 1 kin +0.05 IAA	12	366	
QL + 2 kin +0.05 IAA	12	504	
QL + 0.5 BA +0.05 IAA	12	515.5	
QL + 1 BA +0.05 IAA	12	730	
QL + 2 BA +0.05 IAA	12	827	
QL + 0.5 Z +0.05 IAA	12	786.5	
QL + 1 Z +0.05 IAA	12	814	
QL + 2 Z +0.05 IAA	12	992.5	
Ql control	12	865	
H	24.30999	N	120
Degrees Of Freedom	9	p-level	0.003837
H (corrected)	25.01479		
Median Test			
Overall Median	0.7	Chi-square	28
p-level	0.000954		

- Appendix Table 10: Analysis of variance (Kruskal-Wallis ANOVA) for number of leaves at different auxin levels after six weeks of *in vitro* rooting of mother stock of *C. aronia* in flasks.

Comparing Multiple Independent Samples			
Sample size	Sum of Ranks		
control	12	827.5	
QL +0.5 NAA	12	666	
QL +1.0 NAA	12	728	
QL +1.5 NAA	12	590	
QL +0.5 IBA	12	538.5	
QL +1.0 IBA	12	637	
QL +1.5 IBA	12	524.5	
QL +0.5 IAA	12	1004.5	
QL +1.0 IAA	12	996.5	
QL +1.5 IAA	12	747.5	
H	18.40747245	N	120
Degrees Of Freedom	9	p-level	0.03073
H (corrected)	18.63623518		
Median Test			
Overall Median	8	Chi-square	20.66667
p-level	0.014215585		

- Appendix Table 11: Analysis of variance (Kruskal-Wallis ANOVA) for number of shoots at different auxin levels after six weeks of *in vitro* rooting of mother stock of *C. aronia* in flasks.

Comparing Multiple Independent Samples			
Sample size	Sum of Ranks		
control	12	729.5	
QL +0.5 NAA	12	733.5	
QL +1.0 NAA	12	733.5	
QL +1.5 NAA	12	672	
QL +0.5 IBA	12	733.5	
QL +1.0 IBA	12	855	
QL +1.5 IBA	12	672	
QL +0.5 IAA	12	672	
QL +1.0 IAA	12	787	
QL +1.5 IAA	12	672	
H	2.218113	N	120
Degrees Of Freedom	9	p-level	0.98753
H (corrected)	10.63911		
Median Test			
Overall Median	1	Chi-square	89.66667
p-level	0		

- Appendix Table 12: Analysis of variance (Kruskal-Wallis ANOVA) for shoot height at different auxin levels after six weeks of *in vitro* rooting of mother stock of *C. aronia* in flasks.

Comparing Multiple Independent Samples			
Sample size	Sum of Ranks		
control	12	941.5	
QL +0.5 NAA	12	865.5	
QL +1.0 NAA	12	737	
QL +1.5 NAA	12	413	
QL +0.5 IBA	12	467	
QL +1.0 IBA	12	615.5	
QL +1.5 IBA	12	481	
QL +0.5 IAA	12	918	
QL +1.0 IAA	12	961.5	
QL +1.5 IAA	12	860	
H	28.48395	N	120
Degrees Of Freedom	9	p-level	0.000791
H (corrected)	29.16585		
Median Test			
Overall Median	0.7	Chi-square	38.66667
p-level	1.32E-05		

- Appendix table 13: Analysis of variance Two-Way ANOVA for the effect of media types together with different light conditions on callus-induced from cotyledons- growth.

Summary			
Response	weight		
Factor #1	Media type	Fixed	1 = MS, 2= B5
Factor #2	Light condition	Fixed	1 = Light, 2= Dark

Descriptive Statistics					
Factor	Group	Sample size	Mean	Variance	Standard Deviation
Media type x Light condition	1 x 1	16	0.327231	0.011468	0.1071
Media type x Light condition	1 x 2	16	0.746663	0.071274	0.267
Media type x Light condition	2 x 1	16	0.182406	0.009011	0.0949
Media type x Light condition	2 x 2	16	0.981613	0.067601	0.26
Media type	1	32	0.536947	0.085436	0.2923
Media type	2	32	0.582009	0.201904	0.4493
Light condition	1	32	0.254819	0.015322	0.1238
Light condition	2	32	0.864138	0.081443	0.2854

ANOVA							
Source of Variation	SS	d.f.	MS	F	p-level	F crit	Omega Sqr.
Factor #1 (Media type)	0.0325	1	0.0325	0.81554	0.3701	4.0012	0
Factor #2 (Light condition)	5.9403	1	5.9403	149.109	0	4.0012	0.6571
Factor #1 + #2 (Media type x Light condition)	0.5769	1	0.5769	14.4813	0.0003	4.0012	0.0598
Within Groups	2.3903	60	0.0398				
Total	8.94	63	0.1419				
Omega squared for combined effect	0.7161						

Comparisons among groups of Factor 1 (Media type) within each Factor 2 (Light condition) level				
Factor 1 (Media type) group 1 comparisons				
Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	-0.419431	5.9437	0	accepted
Factor 1 (Media type) group 2 comparisons				
Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	-0.799206	11.325	0	accepted

Comparisons among groups of Factor 2 (Light condition) within each Factor 1 (Media type) level				
Factor 2 (Light condition) group 1 comparisons				
Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	0.144825	2.0523	0.0442	rejected
Factor 2 (Light condition) group 2 comparisons				
Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	-0.23495	3.3294	0.0014	accepted

- Appendix table 14: Analysis of variance Two-Way ANOVA for the effect of media types together with different light conditions on callus-induced from leaves- growth.

Summary			
Response	weight		
Factor #1	media type	Fixed	MS=1 , B5= 2
Factor #2	light condition	Fixed	light= 1 , Dark = 2

Descriptive Statistics					
Factor	Group	Sample size	Mean	Variance	Standard Deviation
media type x light condition	1 x 1	16	0.310481	0.010142	0.10071
media type x light condition	1 x 2	16	0.789	0.075823	0.275361
media type x light condition	2 x 1	16	0.229163	0.005129	0.071614
media type x light condition	2 x 2	16	0.742294	0.045443	0.213174
media type	1	32	0.549741	0.100688	0.317314
media type	2	32	0.485728	0.09242	0.304006
light condition	1	32	0.269822	0.009096	0.095371
light condition	2	32	0.765647	0.05924	0.243394

ANOVA							
Source of Variation	SS	d.f.	MS	F	p-level	F crit	Omega Sqr.
Factor #1 (media type)	0.065562	1	0.065562	1.920688	0.17091	4.001191	0.005164
Factor #2 (light condition)	3.933479	1	3.933479	115.2349	0	4.001191	0.640704
Factor #1 + #2 (media type x light condition)	0.004792	1	0.004792	0.140389	0.709216	4.001191	0
Within Groups	2.048066	60	0.034134				
Total	6.051899	63	0.096062				
Omega squared for combined effect	0.641046						

Comparisons among groups of Factor 1 (media type) within each Factor 2 (light condition) level				
Factor 1 (media type) group 1 comparisons				
Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	-0.47852	7.325674	0	accepted
Factor 1 (media type) group 2 comparisons				
Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	-0.51313	7.855559	0	accepted

Comparisons among groups of Factor 2 (light condition) within each Factor 1 (media type) level				
Factor 2 (light condition) group 1 comparisons				
Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	0.081319	1.244914	0.217703	rejected
Factor 2 (light condition) group 2 comparisons				
Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	0.046706	0.715029	0.477191	rejected

- Appendix table 15: Analysis of variance Two-Way ANOVA for the effect of media types together with different light conditions on callus-induced from roots- growth.

Summary			
Response	weight		
Factor #1	media type	Fixed	1= MS, 2= B5
Factor #2	light condition	Fixed	1= Light, 2= Dark

Descriptive Statistics					
Factor	Group	Sample size	Mean	Variance	Standard Deviation
media type x light condition	1 x 1	16	0.481731	0.078581	0.280324
media type x light condition	1 x 2	16	0.767156	0.147592	0.384176
media type x light condition	2 x 1	16	0.48	0.087487	0.295781
media type x light condition	2 x 2	16	0.79685	0.081754	0.285926
media type	1	32	0.624444	0.130462	0.361196
media type	2	32	0.638425	0.107799	0.328327
light condition	1	32	0.480866	0.080356	0.283472
light condition	2	32	0.782003	0.111201	0.333468

ANOVA							
Source of Variation	SS	d.f.	MS	F	p-level	F crit	Omega Sqr.
Factor #1 (media type)	0.003128	1	0.003128	0.031639	0.859422	4.001191	0
Factor #2 (light condition)	1.450941	1	1.450941	14.67772	0.000308	4.001191	0.180566
Factor #1 + #2 (media type x light condition)	0.00395	1	0.00395	0.039959	0.842236	4.001191	0
Within Groups	5.931198	60	0.098853				
Total	7.389216	63	0.117289				
Omega squared for combined effect	0.155108						

Comparisons among groups of Factor 1 (media type) within each Factor 2 (light condition) level				
Factor 1 (media type) group 1 comparisons				
Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	-0.28543	2.567683	0.012584	accepted
Factor 1 (media type) group 2 comparisons				
Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	-0.31685	2.850382	0.00587	accepted

Comparisons among groups of Factor 2 (light condition) within each Factor 1 (media type) level				
Factor 2 (light condition) group 1 comparisons				
Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	0.001731	0.015574	0.987622	rejected
Factor 2 (light condition) group 2 comparisons				
Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	-0.02969	0.267125	0.790232	rejected

- Appendix Table 16: Analysis of variance (ANOVA) for callus-induced from cotyledons-fresh weight at eight different media types after four weeks of culture of callus clumps.

Summary				
Groups	Sample size	Sum	Mean	Variance
B5+0.1TDZ+0.1NAA	24	7.8806	0.328358	2.701864
B5+0.5TDZ+0.1NAA	24	10.1483	0.422846	4.688267
B5+0.5TDZ+0.5NAA	24	14.9557	0.623154	9.987759
B5+1.0TDZ+0.5NAA	24	17.0051	0.708546	12.56937
B5+0.1 BA+0.1NAA	24	12.0984	0.5041	6.307846
B5+0.5 BA+0.1NAA	24	13.7608	0.573367	8.204758
B5+0.5 BA+0.5NAA	24	13.4939	0.562246	7.799398
B5+1.0 BA+0.5NAA	24	12.6348	0.52645	6.970474
Total	192		0.531133	0.026524

ANOVA							
Source of Variation	d.f.	SS	MS	F	p-level	F crit	Omega Sqr.
Between Groups	7	2.310991	0.330142	22.04906	0	2.059637	0.434202
Within Groups	184	2.75504	0.014973				
Total	191	5.066031					

Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	-0.0945	2.6749	0.0081	Accepted
1 vs 3	-0.2948	8.3456	0	Accepted
1 vs 4	-0.3802	10.763	0	Accepted
1 vs 5	-0.1757	4.9752	0	Accepted
1 vs 6	-0.245	6.9361	0	Accepted
1 vs 7	-0.2339	6.6213	0	Accepted
1 vs 8	-0.1981	5.6079	0	Accepted
2 vs 3	-0.2003	5.6707	0	Accepted
2 vs 4	-0.2857	8.0881	0	Accepted
2 vs 5	-0.0813	2.3003	0.0225	Accepted
2 vs 6	-0.1505	4.2612	0	Accepted
2 vs 7	-0.1394	3.9464	0.0001	Accepted
2 vs 8	-0.1036	2.933	0.0038	Accepted
3 vs 4	-0.0854	2.4174	0.0166	Accepted
3 vs 5	0.1191	3.3704	0.0009	Accepted
3 vs 6	0.0498	1.4095	0.1604	Rejected
3 vs 7	0.0609	1.7243	0.0863	Rejected
3 vs 8	0.0967	2.7377	0.0068	Accepted
4 vs 5	0.2044	5.7878	0	Accepted
4 vs 6	0.1352	3.8269	0.0002	Accepted
4 vs 7	0.1463	4.1417	0	Accepted
4 vs 8	0.1821	5.1551	0	Accepted
5 vs 6	-0.0693	1.9609	0.0514	Rejected
5 vs 7	-0.0581	1.6461	0.1014	Rejected
5 vs 8	-0.0224	0.6327	0.5277	Rejected
6 vs 7	0.0111	0.3148	0.7532	Rejected
6 vs 8	0.0469	1.3282	0.1857	Rejected
7 vs 8	0.0358	1.0134	0.3122	Rejected

- Appendix Table 17: Analysis of variance (ANOVA) for callus-induced from leaves-fresh weight at eight different media types after four weeks of culture of callus clumps.

Summary				
Groups	Sample size	Sum	Mean	Variance
MS+ 0.1TDZ+0.1NAA	24	12.5856	0.5244	6.896254
MS +0.5TDZ+0.1NAA	24	12.5367	0.522363	6.814701
MS+ 0.5TDZ+0.5NAA	24	18.2322	0.759675	14.25851
MS +1 TDZ +0.5NAA	24	18.6326	0.776358	15.1639
MS+0.1BA+0.1NAA	24	8.2251	0.342713	2.991597
MS+0.5BA+0.1NAA	24	10.2462	0.426925	5.026797
MS+0.5BA+0.5NAA	24	13.1523	0.548013	8.160942
MS+1BA+0.5NAA	24	11.5907	0.482946	6.207271
Total	192		0.547924	0.041244

ANOVA							
Source of Variation	d.f.	SS	MS	F	p-level	F crit	Omega Sqr.
Between Groups	7	3.820852	0.545836	24.7572	0	2.059637	0.464137
Within Groups	184	4.056753	0.022048				
Total	191	7.877605					

Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	0.002	0.0475	0.9621	rejected
1 vs 3	-0.2353	5.4889	0	accepted
1 vs 4	-0.252	5.8781	0	accepted
1 vs 5	0.1817	4.2387	0	accepted
1 vs 6	0.0975	2.2741	0.0241	accepted
1 vs 7	-0.0236	0.5509	0.5824	rejected
1 vs 8	0.0415	0.9671	0.3347	rejected
2 vs 3	-0.2373	5.5364	0	accepted
2 vs 4	-0.254	5.9257	0	accepted
2 vs 5	0.1797	4.1912	0	accepted
2 vs 6	0.0954	2.2265	0.0272	rejected
2 vs 7	-0.0257	0.5984	0.5503	rejected
2 vs 8	0.0394	0.9196	0.359	rejected
3 vs 4	-0.0167	0.3892	0.6976	rejected
3 vs 5	0.417	9.7276	0	accepted
3 vs 6	0.3328	7.763	0	accepted
3 vs 7	0.2117	4.938	0	accepted
3 vs 8	0.2767	6.456	0	accepted
4 vs 5	0.4336	10.117	0	accepted
4 vs 6	0.3494	8.1522	0	accepted
4 vs 7	0.2283	5.3273	0	accepted
4 vs 8	0.2934	6.8452	0	accepted

5 vs 6	-0.0842	1.9647	0.0509	rejected
5 vs 7	-0.2053	4.7896	0	accepted
5 vs 8	-0.1402	3.2716	0.0013	accepted
6 vs 7	-0.1211	2.8249	0.0052	accepted
6 vs 8	-0.056	1.307	0.1928	rejected
7 vs 8	0.0651	1.518	0.1307	rejected

- Appendix Table 18: Analysis of variance (ANOVA) for callus of red line fresh weight at three different media types after four weeks of culture of callus clumps.

Summary				
Groups	Sample size	Sum	Mean	Variance
B5+0.5 2,4-D	24	57.6338	2.401408	144.3794
B5+2mg/l 2,4-D+1.5mg/l kin	24	56.3327	2.347196	135.4051
MS+2mg/l 2,4-D+1.5mg/l kin	24	58.1488	2.422867	152.3345
Total	72		2.39049	0.291254

ANOVA							
Source of Variation	d.f.	SS	MS	F	p-level	F crit	Omega Sqr.
Between Groups	2	0.073	0.0365	0.1222	0.8851	3.1296	-0.025
Within Groups	69	20.61	0.2986				
Total	71	20.68					

Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	0.0542	0.3437	0.7321	rejected
1 vs 3	-0.0215	0.136	0.8922	rejected
2 vs 3	-0.0757	0.4797	0.6329	rejected

- Appendix Table 19: Analysis of variance (ANOVA) for callus -induced from cotyledons-fresh weight that were cultured on B5 supplemented with 0.5 mg/L 2,4-D in response to different antioxidant treatment after four weeks of culture.

Summary				
Groups	Sample size	Sum	Mean	Variance
B5+0.5 2,4-D +1g/l PVP	16	11.1947	0.699669	9.043858
B5 +0.5 2,4-D + 2g/L charcol	16	6.5501	0.409381	2.867395
B5+0.5 2,4-D + citric acid	16	6.6941	0.418381	3.016281
B5+ 0.5 2,4-D + ascorbic acid	16	5.8009	0.362556	2.202402
Total	64		0.472497	0.045107

ANOVA							
Source of Variation	d.f.	SS	MS	F	p-level	F crit	Omega Sqr.
Between Groups	3	1.1297	0.3766	13.1972	0	2.7581	0.3638
Within Groups	60	1.712	0.0285				
Total	63	2.8417					

Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	0.2903	4.8606	0	accepted
1 vs 3	0.2813	4.7099	0	accepted
1 vs 4	0.3371	5.6447	0	accepted
2 vs 3	-0.009	0.1507	0.8807	rejected
2 vs 4	0.0468	0.784	0.436	rejected
3 vs 4	0.0558	0.9347	0.3535	rejected

- Appendix Table 20: Analysis of variance (ANOVA) for red colored callus fresh weight that were cultured on B5 supplemented with 0.5 mg/L 2,4-D in response to different antioxidant treatment after four weeks of culture.

Summary				
Groups	Sample size	Sum	Mean	Variance
B5+0.5 2,4-D +1g/l PVP	16	23.8884	1.493025	36.82285
B5 + 0.5 2,4-D +2g/L Charcol	16	14.0825	0.880156	13.63529
B5 + 0.5 2,4-D +citric acid	16	13.964	0.87275	13.58585
B5 + 0.5 2,4 -D + ascorbic acid	16	12.3058	0.769113	10.57397
Total	64		1.003761	0.160883

ANOVA							
Source of Variation	d.f.	SS	MS	F	p-level	F crit	Omega Sqr.
Between Groups	3	5.2301	1.7434	21.3232	0	2.7581	0.4879
Within Groups	60	4.9056	0.0818				
Total	63	10.136					

Fisher LSD				
Group vs Group	Difference	Test Statistics	p-level	Accepted?
1 vs 2	0.6129	6.0624	0	accepted
1 vs 3	0.6203	6.1357	0	accepted
1 vs 4	0.7239	7.1608	0	accepted
2 vs 3	0.0074	0.0733	0.9418	rejected
2 vs 4	0.111	1.0984	0.2763	rejected
3 vs 4	0.1036	1.0252	0.3093	rejected

- Appendix Table 21: 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
Agar European Bacteriological	Hy labs	5090076
Chloroform	Frutarom	5551020
2,4-Dichlorophenolxyacetic acid	Sigma	125k0703
70% ethanol	Chatham	7408/10
Gamborg B5 medium including vitamins	Duchefa biochemie	G0210.0010
Gibberellic acid	Duchefa biochemie	60907.0005
Indole-3-acetic acid	Duchefa biochemie	10901.0025
Indole-3-butyric acid	Sigma	105k1151
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
Sucrose	Duchefa biochemie	S0809.1000
Thidiazuron	Duchefa biochemie	51707-55-2
Zeatin	Duchefa biochemie	16.37.36-4
Quoirin and Lepoivre Basal Salt Mixture	Duchefa biochemie	Q025.0050
Kinetin	Duchefa biochemie	K0905.0005