إقـــرار

أنا الموقع أدناه مقدم الرسالة التي تحمل العنوان:

***In vitro*, Micropropagation of an Apple *Malus × domestica* Borkh. (Cultivar Anna) by Cloning of Apical and Axillary Shoot Buds**

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In vitro, Micropropagation of an Apple Malus × domestica Borkh. (Cultivar Anna) by Cloning of Apical and Axillary Shoot Buds

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الإكثار الدقيق مخبرياً لنبات التفاح Malus X domestica من الصنف Anna بزراعة البراعم القمية والإبطية

In Vitro, Micropropagation of an apple Malus X domestica Borkh. (Cultivar Anna) by cloning of Apical and Axillary Shoot Buds

وبعد المناقشة العلنية التي تمت اليوم الاثنين 20 ربيع الآخر 1436هـ، الموافق 2015/02/09م الساعة الثانية والنصف مساءً بمبنى طيبة، اجتمعت لجنة الحكم على الأطروحة والمكونة من:

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واللجنة إذ تمنحها هذه الدرجة فإنها توصيها بتقوى الله ولزوم طاعته وأن تسخر علمها في خدمة دينها ووطنها.

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Dedication

I dedicate this thesis to my husband who supported my research and helped me to achieve this degree.

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Abstract

***In vitro,* Micropropagation of an Apple *Malus* × *domestica* Borkh. (Cultivar Anna) by Cloning of Apical and Axillary Shoot Buds**

Apple plant *(Malus* x *domestica)* is perennial fruit tree belonging to the family *Rosaceae.* Apple species have wide geographical distribution. Apple fruit has been considered to have high health benefits. Most of the species intercross. Since self-incompatibility is common, apples produced from seed are mostly interspecific hybrids, therefore it is difficult to produce true-to-type cultivars from seeds. The objective of our study, therefore, was to develop micropropagation protocol for Anna apple (*Malus domestica* Borkh), which is highly adapted for environmental conditions of Gaza Strip, using buds explants on MS medium. Buds were collected during the summer season produced higher percentage of buds establishment (75%) as compared to buds collected during other seasons. The best sterilization protocol was when we used 5% NaOCl, Tween 20, 70% Alcohol and 0.5% $HgCl_2$. Apple buds (3.0 mm in length) were established by using AC, AA and CA as antioxidants. In culture establishment we used full strength MS media with several combinations of hormones BAP, GA3 and IBA. We obtained the best results in group B2 (1mg/l BAP, 0.5mg/l GA3 and 0.1mg/l IBA). The highest establishment rate was obtained for explant that produced 6.40 ± 1.14 shoots (1.76 ± 0.34 cm in length). In multiplication stage, we obtained the best results in group H1 (1mg/l BAP, 0.5mg/l GA3 and 0.1mg/l IBA). The highest multiplication rate was obtained for explant that produced 6.80 ± 3.03 shoots (1.88 ± 0.62 cm in length). In rooting stage we used two auxins IBA or IAA, the roots were formed only in two samples when we used 1 mg/l IBA and 0.1 mg/l IAA in half strength MS solid media.

This research is considered the first in Gaza Strip for *in vitro* micropropagation of woody plant by using apple shoot buds.

Key words: Anna apple, Plant hormones, micropropagation, establishment stage, multiplication stage, rooting stage.

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الملخص

الإكثار الدقيق مخبرياً لنبات التفاح *Malus x domestica* من الصنف Anna بزراعة البراعم القمية والإبطية

نبات التفاح *Malus x domestica* هو من أشجار الفاكهة المعمرة والتي تنتمي إلى العائلة الوردية. أنواع التفاح لها توزيع جغرافي واسع. فاكهة التفاح لها فائدة صحية عالية، معظم الأنواع ذاتية التلقيح. بالرغم من أن التوافق الذاتي هو الشائع، التفاح المنتج من البذور هو في الغالب أنواع هجينة، وبالتالي يصعب الحصول على صنف صحيح النوع من البذور. لذلك كان هدف هذه الدراسة وضع بروتوكول للإكثار الدقيق لتفاح الآنا، الذي يتكيف بشكل كبير في الظروف البيئية في قطاع غزة، وذلك باستخدام البراعم على بيئة (MS). البراعم التي تم جمعها خلال فصل الصيف أعطت أعلى نسبة في تأسيس البراعم (75%) مقارنة بالبراعم التي تم جمعها في فصول السنة الأخرى. بروتوكول التعقيم الأفضل كان باستخدام هيبوكلورات الصوديوم 5% و توين 20 و كحول إيثيلي 70% و كلوريد الزئبق 0.5 %. براعم التفاح تم تأسيسها باستخدام AC و AA و CA كمضادات للأكسدة. في بيئة التأسيس استخدمنا بيئة (MS) كاملة التركيز وبإضافة العديد من التراكيز الهرمونية BAP و GA3 و IBA . حصلنا على أفضل نتيجة في المجموعة B2 باستخدام (1مليجرام/ لتر BAP، 0.5 مليجرام/ لتر GA3 مع 0.1 مليجرام/ لتر IBA). أعلى معدل للتأسيس حصلنا عليه للعينات النباتية التي أعطت (1.14±6.40) shoots و كان الطول (0.34±1.76 سم). في مرحلة الإكثار، حصلنا على أفضل النتائج في المجموعة H1 (1مليجرام/ لتر BAP، 0.5 مليجرام/ لتر GA3 مع 0.1 مليجرام/ لتر IBA) . أعلى معدل للإكثار حصلنا عليه للعينات النباتية التي أعطت

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(3.03±6.80) shoots و كان الطول (1.88±0.62 سم). وفي مرحلة التجذير تم استخدام نوعين من الأوكسينات IBA أو IAA تكونت الجذور في عينتين فقط وذلك عند استخدام كل من IBA بتركيز 1ملجرام/لتر و IAA بتركيز 0.1 ملجرام/لتر في بيئة (MS) صلبة بنصف التراكيز.

وتعتبر الدراسة مبادرة هي الأولى من نوعها للإكثار مخبريا لنبات خشبي باستخدام براعم تفاح الآنا في قطاع غزة.

الكلمات المفتاحية: تفاح الآنا، الهرمونات النباتية، الإكثار الدقيق، مرحلة التأسيس، مرحلة التكثير، مرحلة التجذير.

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Table of contents

Dedication i

Acknowledgment ii

Abstract iii

الملخص iv

Table of contents vi

List of tables xii

List of figures xv

List of abbreviations xvii

Chapter 1: Introduction 1

1.1 Plant tissue culture 1

1.2 The Apple 2

1.3 Apple *Malus* × *domestica* Borkh. cultivar Anna 3

1.4 General objective 3

1.4.1 Specific Objectives 3

1.5 Significance 4

Chapter 2: Literature review.. 5

2.1 Apple species 5

2.2 Apple cultivars 6

المنارة للاستشارات

www.manaraa.com

2.3 Description of *Malus* x *domestica* Borkh. 6

2.4 Health benefits of using apple 7

2.5 Advantage of micropropagation 9

2.6 Methods of tissue culture 10

2.7 Elimination of the browning 10

2.8 Stages of micropropagation 11

2.9 Previous studies 12

2.9.1 Important of hormones in apple tissue culture 12

2.9.2 Importance of sucrose and Sodium chloride concentration on apple tissue culture 16

Chapter 3: Materials and methods 18

3.1 Materials 18

3.1.1 Chemicals 18

3.1.2 Equipments 20

3.2 Methods 20

3.2.1 Stock plant preparation 20

3.2.2 Stock solution preparation 20

3.2.3 Culture media preparation 21

3.2.4 Surface Sterilization of Explants 21

3.2.5 Establishment stage 21

المنارة للاستشارات

www.manaraa.com

1. Pilot experiment 22
2. First experiment 22
3. Second experiment 22
4. Third experiment 22
5. Fourth experiment 23
6. Fifth experiment 24
7. Sixth experiment 24
3.2.6 Shoot multiplication 24
1. Pilot experiment 24
2. First experiment 24
3. Second experiment 24
4. Third experiment 25
5. Fourth, fifth, and sixth experiments 25
3.2.7 Rooting stage 26
1. Pilot experiment 26
2. First experiment 26
3. Second experiment 26
4. Third experiment 26
5. Fourth, fifth and sixth experiments 27

المنارة للاستشارات
www.manaraa.com

3.2.8 Experimental design and statistical data analysis 27

Chapter 4: Results 28

4.1 Surface Sterilization of Explants 28

4.2 Establishment stage 30

1. Pilot experiment 31

2. First experiment 31

3. Second experiment 31

4. Third experiment 45

5. Forth experiment 50

6. Fifth experiment (Callus stage) 50

7. Sixth experiment 51

4.3 Shoot multiplication 51

1. Pilot experiment 51

2. First experiment 51

3. Second experiment 51

4. Third experiment 57

5. Fourth and fifth experiments 57

6. Sixth experiment 57

4.4 Rooting stage 57

المنارة للاستشارات

www.manaraa.com

1. Pilot and First experiments 57

2. Second experiment 57

3. Third experiment 58

4. Fourth, fifth and sixth experiments 59

Chapter 5: Discussion 60

5.1 Effect of the season on the percentage of contamination of samples 60

5.2 Surface Sterilization of Explants 61

5.3 Phenolic browning 61

5.4 Establishment stage 62

1. Pilot experiment 62

2. First experiment 62

3. Second experiment 62

4. Third experiment 63

5. Fourth experiment 63

6. Fifth experiment 64

7. Sixth experiment 64

5.5 Shoot multiplication 64

1. Pilot experiment 64

2. First experiment 64

المنارة للاستشارات

www.manaraa.com

3. Second experiment 65

4. Third experiment 65

5. Fourth experiment 65

6. Fifth experiment 65

7. Sixth experiment 66

5.6 Rooting stage 66

1. Pilot experiment 66

2. First experiment 66

3. Second experiment 66

4. Third experiment 67

5. Fourth, fifth and sixth experiment 67

6. The difficulties faced this research 68

Chapter 6: Conclusion and Recommendation 69

6.1 Conclusion 69

6.2 Recommendations 70

Chapter 7: References 71

Appendix 79

المنارة للاستشارات
www.manaraa.com

List of tables

No. of Table	Title	Page
Table 2.1	Apple fruit nutritional value per 100 g. Nutrient Database, 2008	8
Table 3.1	list of the chemicals used in this work	18
Table 3.2	list of the main equipments used in this work	20
Table 3.3	Hormones concentrations in 10 groups which used in establishment stage	23
Table 3.4	Hormones concentrations in 3 groups which used in multiplication stage	25
Table 4.1	Effect of surface sterilization treatments and seasons on contamination of *in vitro* bud culture of Anna (after one week)	29
Table 4.2	Statistical analysis for establishment stage in autumn after one week (group A)	32
Table 4.3	Statistical analysis for establishment stage in autumn after two weeks (group A)	33
Table 4.4	Statistical analysis for establishment stage in autumn after three weeks (group A)	34
Table 4.5	Statistical analysis for establishment stage in autumn after four weeks (group A)	35
Table 4.6	Statistical analysis for establishment stage in autumn after one week (group B)	36
Table 4.7	Statistical analysis for establishment stage in autumn after two weeks (group B)	37

المنارة للاستشارات

www.manaraa.com

Table 4.8	Statistical analysis for establishment stage in autumn after three weeks (group B)	38
Table 4.9	Statistical analysis for establishment stage in autumn after four weeks (group B)	39
Table 4.10	Statistical analysis for establishment stage in autumn after one week (group C)	40
Table 4.11	Statistical analysis for establishment stage in autumn after two weeks (group C)	41
Table 4.12	Statistical analysis for establishment stage in autumn after three weeks (group C)	42
Table 4.13	Statistical analysis for establishment stage in autumn after four weeks (group C)	43
Table 4.14	Statistical analysis for comparison between A, B and C groups in establishment stage after four weeks	44
Table 4.15	Statistical analysis for establishment stage in winter after one week (group B)	45
Table 4.16	Statistical analysis for establishment stage in winter after two weeks (group B)	46
Table 4.17	Statistical analysis for establishment stage in winter after three weeks (group B)	47
Table 4.18	Statistical analysis for establishment stage in winter after four weeks (group B)	48
Table4.19	Statistical analysis for comparison between group B in Autumn and group B in Winter in establishment stage after four weeks	49

المنارة للاستشارات
www.manaraa.com

Table 4.20	Statistical analysis for multiplication stage after one week	52
Table 4.21	Statistical analysis for multiplication stage after two weeks	53
Table 4.22	Statistical analysis for multiplication stage after three weeks	54
Table 4.23	Statistical analysis for multiplication stage after four weeks	55

المنارة للاستشارات
www.manaraa.com

List of figures

No. of figure	Title	Page
Figure 2.1	Apple flower and fruit morphology	7
Figure 4.1	Establishment stage (A. after planting B. after 2 weeks C. after 4weeks)	31
Figure 4.2	Establishment stage in autumn after one week (group A)	32
Figure 4.3	Establishment stage in autumn after two weeks (group A)	33
Figure 4.4	Establishment stage in autumn after three weeks (group A)	34
Figure 4.5	Establishment stage in autumn after four weeks (group A)	35
Figure 4.6	Establishment stage in autumn after one week (group B)	36
Figure 4.7	Establishment stage in autumn after two weeks (group B)	37
Figure 4.8	Establishment stage in autumn after three weeks (group B)	38
Figure 4.9	Establishment stage in autumn after four weeks (group B)	39
Figure 4.10	Establishment stage in autumn after one week (group C)	40
Figure 4.11	Establishment stage in autumn after two weeks (group C)	41
Figure 4. 12	Establishment stage in autumn after three weeks (group C)	42
Figure 4.13	Establishment stage in autumn after four weeks (group C)	43
Figure 4.14	Comparison between A, B and C groups in establishment stage after four weeks	44
Figure 4.15	Establishment stage in winter after one week (group B)	45
Figure 4.16	Establishment stage in winter after two weeks (group B)	46
Figure 4.17	Establishment stage in winter after three weeks (group B)	47

المنارة للاستشارات

www.manaraa.com

Figure 4.18	Establishment stage in winter after four weeks (group B)	48
Figure 4.19	Comparison between group B in Autumn and group B in Winter in establishment stage after four weeks	49
Figure 4.20	Callus inductions after one month in establishment media	50
Figure 4.21	Multiplication stage after one week	52
Figure 4.22	Multiplication stage after two week	53
Figure 4.23	Multiplication stage after three weeks	54
Figure 4.24	Multiplication stage after four weeks	55
Figure 4.25	Multiplication stage in H1 media (A, B, C, D and E)	56
Figure 4.26	Multiplication stage in H2 media (F, G, H, I and J)	56
Figure 4.27	Rooting stage (A. after 5 days B. after 2weeks C. after 1 month)	58
Figure 4.28	Rooting stage (after 1 month)	59

المنارة للاستشارات
www.manaraa.com

List of Abbreviations

Abbreviation	The term
AC	Activated Charcoal
ANOVA	Analysis Of Variance
AA	Ascorbic Acid
BAP	6-Benzyl Amino Purine
CA	Citric Acid
2,4-D	2,4-Dichlorophenoxyacetic Acid
GA3	Gibberlic Acid
$HgCl_2$	Mercuric Chloride
IAA	Indole Acetic Acid
IBA	Indole-3-Butyric Acid
KIN	Kinetin
MS	Murashige and Skoog
NAA	Naphthalene Acetic Acid
NaOCl	Sodium Hypochlorite
POX	Peroxidases
PGRs	Plant Growth Regulators
PPO	Polyphenol Oxidase
PVP	Polyvinyl Pyrolidone
QL	Quoirin and Lepoivre
TDZ	Thidiazuron

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Chapter 1

Introduction

1.1 Plant tissue culture

Plant tissue culture has emerged as a powerful technique in plant propagation, particularly in the production of virus-free plants of high- value varieties as well as in genetic transformation studies (Premkumar *et al*., 2001).

Historically, the science of tissue culture development is linked to the discovery of cell and subsequent cell theory, which states that the cell is the basic structural unit of all living things. In 1902, the German botanist Gottlieb Haberlandt developed the concept of *in vitro* cell culture. He isolated single cells from palisade tissue of leaves, pith parenchyma, epidermis and epidermal hair of various plants and cultured on Knop's salt solution containing glucose and peptone. In his cultures, cells that synthesized starch and increased in size survived for several weeks though none of them divided. He predicted the requirements for cell division under experimental conditions that have been proved through time. Therefore, Haberlandt is considered as the father of plant tissue culture. Following Haberlandt, many workers continued working on plant tissue cultures. In 1939, Gautheret cultivated cambial tissues of carrot root, Nobecourt (carrot), and White (tobacco) for prolonged periods of time. In strict sense, these were the first true plant tissue cultures (Chawla, 2002).

Propagation of woody plants by conventional methods necessarily limits the rate of output and makes the end product expensive. Tissue culture can overcome this problem since it has been reported that may acquire higher rooting capability after continuously subculturing *in vitro*. Tissue culture techniques provide a fast and dependable method for the production of a large quantity of uniform plantlets in a short time throughout the year. Micropropagated plants from both the cultures of performed structures, such as shoot tips and axillary buds and from the tissues of

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hardwood shoot cuttings have been reported to maintain clonal fidelity (Boudabous *et al*., 2010).

In vitro plant regeneration from the apical/axillary shoot buds and nodal explants has been reported in a number of varieties of apple. Micropropagation of apple to produce self-rooted plants will open up new areas of research and will allow changes in traditional fruit tree propagation. So far, apple micropropagation has been attempted with only varying success. It has been reported that different cultivars do not respond in the same way during establishment, proliferation and rooting *in vitro* (Boudabous *et al*., 2010).

1.2 The Apple

Apple (Malus sp., family - Rosaceae) is an important temperate fruit crop and it is second in production among temperate fruit crops grown worldwide. A large number of currently grown commercial apple cultivars are hybrid selection and have been derived as chance seedling (Rai *et al*., 2009).

The word apple comes from the old English word "aeppel", which has relation with a number of Indo-European language families. The prevailing theory is that "apple" may be one of the most ancient Indo-European words "*abl"* to come down to English in a recognizable form (Dobrzanski *et al*., 2006).

Micropropagation allows quick propagation of new varieties or breeding lines or variants for apple breeders. It is an essential step in the success of regeneration of transgenic lines and determines the effectiveness of a transformation protocol. The history of apple tissue culture dates back to the late 1960s and the early 1970s when apple shoots had been cultured *in vitro* and their growth was first reported. Since then many genotypes have been successfully cultured *in vitro*, and a number of papers have been published on different aspects of apple micropropagation (Dobranszki, and Teixeira da Silva 2010).

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1.3 Apple *(Malus × domestics* Borkh.) cultivar Anna

Apple *(Malus × domestica* Borkh.) cultivar Anna developed in 1959 in "Israel" from a cross between "Golden Delicious" and "Red Hadassiya" (Hauagge and Cummins1991)**.** Anna has a low chilling requirement and has one of the few cultivars that are productive with irrigation under hot desert conditions. Fruit development requires 120 days, with the fruit ripening during a period of extremely high day temperatures (> 40º C) **(**Trejo-Gonzalez and Soto, 1991).

Fruit of Anna resembles that of "Red Delicious" more than other low-chill apple cultivars. Fruit of Anna typically attains a size of 2 1/4 - 2 1/2 inches with a 50 percent red blush and good flavor (Andersen and Crocker, 2000). The total apple production in the Gaza Strip up to 632 tons/year, but this quantity reach to 6.5% of the actual requirement in Gaza Strip (Ministry of Agriculture - Palestine, 2015).

1.4 General objective

In vitro micropropagation apple *Malus* × *domestica* Borkh. (cultivar Anna) by apical and axillary shoot buds, using different hormones concentration during different seasons.

1.4.1 Specific Objectives

The specific objectives of the present study were:

1. To investigate different surface sterilization protocols to overcome the contamination during different seasons.
2. To overcome the problem of oxidation and minimize phenolic exudation.
3. To optimize the concentrations of different growth regulators and their combinations on MS medium for optimum production of shoots.
4. To determine the multiplication stage with highest number of multiplied micro-shoots.

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5. To investigate rooting response of shoots to different IBA or IAA concentrations in combination with IBA.

1.5 Significance

1. Gaza Strip depends largely on the agriculture sector, making the agriculture sector one of the main reasons for the development.
2. Apple is considered as one of the plant varieties with high nutritional value, high productivity, easy of marketing, simple agricultural needs and commercially required which makes the process of cultivation and production in commercial quantities is one of the real solutions for the economic problems in Gaza Strip.
3. The blockade and the general political situation require us to look for ways to produce seedlings instead of importing and manner that does not detract from the quality and stability of gene using tissue culture.
4. From this standpoint, the goals of this research are a clear basis contributes to the solution of related problems in agricultural and economic side.

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Chapter 2

Literature Review

2.1 Apple species

Apple, pear, plum, and peach trees belong to the Rosaceae family. Apple and pear, as other genera, have been classified inside Maloideae family because they produce pome type fruits (Bretaudeau and Faure 1991; Janick *et al.* 1996). They are 27 primary apple species together with their origin and use. A total of 22 of 27 species (82%) are from Asia (11 located mainly in China), 4 in North America, 2 in Europe, and 1 in Japan. Six species are used for fruit: M. sieversii, M. sylvestris, M. angustifolia, M. ioensis, M. coronaria, and M. hupehensis. Five out of 27 are recognized as ornamental and 12 as possible rootstocks (Forsline *et al*., 2003). Robinson *et al*. (2001) described that the number of species in genus *Malus* depends upon the rank given to several taxa, species being sub- species and putative hybrids and the nomenclature of the taxa is complex.

The taxonomic hierarchy for the domesticated apple would be:

- Kingdom: Plantae
- Division: Siermalophyta
- Class: Magnoliopsida
- Order: Rosales
- Family: Rosaceae
- Subfamily: Maloideae
- Section: Malus
- Genus: *Malus*
- Species: *M. domestica*

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2.2 Apple cultivars

More than 7,500 cultivars of apple are known to be cultivated. The cultivars vary in size, shape, color, crispness, firmness, texture, juiciness, sweetness and nutritional value of fruit (Pre-Aymard, 2003). Scientific nomenclature for apples has changed since Linnaeus denominated *Pyrus malus*. Other naming in the past have been *M. communis, M. Sylvestris, M. pumila* and *M. domestica* (Harris *et al*., 2002). Mostly the wild apple cultivars are diploid (2n = 34 chromosomes) and some of them are triploid (3n = 51), even some others are tetraploid (4n = 68). Triploids produce relatively heavier sized fruits (Broothaerts *et al.,* 2004). The cultivated apple is likely the result of interspecies hybridization and at present the binomial *Malus x domestica* has been generally accepted as the appropriate scientific name (Korban and Skirvin, 1994). The multiplication sign "X" placed between the genus and species names denotes an interspecific hybridization within the genus (Korban, 1986).

2.3 Description of *Malus* x *domestica* Borkh

Malus x *domestica* Borkh is a small deciduous perennial tree, 5-12 m tall and age longevity between 60 to 100 years. Depending on the rootstock cultivar and the age of the tree, the roots can occupy between 2 to 104 m², most frequently ranging between 10-30 m². The leaves are alternately arranged, simple oval with an acute tip and serrated margin, 5-12 cm long and 3-6 cm broad which are attached on a 2-5 cm petiole (Dobrzanski *et al*., 2006).

The apple tree is a monoecious species with hermaphroditic flowers. Three to six flowers in cymes (the first flower is the most advanced) appear in mixed buds (Dennis, 2003). It produces rose epigynous flowers, sometimes white, with five sepals, petals, and pistils and up to 20 stamens. The development of a multicarpellate inferior ovary (forming the core) and the accessory tissue after fecundation becomes in the fruit known as pome (Ryugo, 1988). The ovary has five carpals at the center of the fruit which are arranged in a five point star, each usually containing one to three ovules, so that in most cases, the maximum seed content is 10 but some cultivars have more. "Liberty" and "Northern Spy", for example, usually have 12 to 18 seeds

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and the "Ottawa 3" rootstock often has 20 to 30 seeds. The fruit matures in autumn, and it is typically 5-8 cm in diameter (rarely up to 15 cm) (Dobrzanski *et al*., 2006).

Apples can grow from seeds. However, like most of perennial fruits, apples are ordinarily propagated asexually by grafting. This is because apple seedlings are examples of extreme heterozygous, in this case rather than inheriting DNA from their parents to create a new apple with characteristics, they are instead different from their parents, sometimes radical (Harries *et al*., 2002). Apples can also form bud sports (mutations on a single branch). Some bud sports turn out to be improved strains of the parent cultivar and others differ sufficiently from the parent tree to be considered new cultivars. Apples are mostly self–incompatible; they must cross-pollinate to develop fruit (Sheffield *et al*., 2005).

Figure 2.1 Apple flower and fruit morphology

2.4 Health benefits of using apple

As indicated by the proverb "an apple a day keeps the doctor away", using apple fruit have been considered to have high health benefit. Different researches suggest that apples can reduce the risk of prostate, colon and lung cancer. Different group of chemicals in apple could protect the brain from neurodegenerative diseases like Alzheimer's and Parkinsonism (Dobrzanski *et al*., 2006). Fruits and vegetables are high in antioxidants, a diet high in these foods helps to prevent oxidative stress, different chronic diseases and slow aging. Apple fruit is very good source of

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important phytochemicals like antioxidants, flavonoids, and other free phenolics which are not bound to other compounds (Boyer and Liu, 2004).

Table 2.1 Apple fruit nutritional value per 100 g USDA (United States Department of Agriculture), Nutrient Database, 2008

Element/Compound	Amount
Carbohydrates	13.81 g
Dietary fiber	2.4 g
Fat	0.17 g
Protein	0.26 g
Vitamin A equiv.	3 μg
Thiamin (Vit. B1)	0.017 mg
Riboflavin (Vit. B2)	0.026 mg
Niacin (Vit. B3)	0.091 mg
Pantothenic acid (B5)	0.061 mg
Vitamin B6	0.041 mg
Folate (Vit. B9)	3 μg
Vitamin C	4.6 mg
Calcium	6 mg
Iron	0.12 mg
Magnesium	5 mg
Phosphorus	11 mg
Potassium	107 mg
Zinc	0.04 mg

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In the year 2010 total world production of fresh apples exceeded (64.3 million t/year) (Dobranszki, and Teixeira da Silva, 2010).

Apples are produced mainly for the fresh market. Specifically in USA, apples are processed into five basic products, viz., juice, canned puree, canned slices, dried apples, and frozen slices. Apple juice and canned sauce are the dominant products (one-half and one-third, respectively). Apples are also processed into vinegar, jelly, apple butter, mincemeat, and fresh slices. Small quantities are also made into apple wine, apple essence, baked whole apples, apple rings, and apple nectar. Another important product is cider, mainly in France, UK, and Spain (Dobrzanski *et al.*, 2006).

2.5 Advantage of micropropagation

Micropropagation confers distinct advantages not possible with conventional propagation method. It is possible to multiply a single explant into several thousands in less than a year. Actively dividing cultures are continuous sources of plantlets without seasonal interruption. It has high commercial potential due to the speed of propagation, clonal propagation, germplasm conservation, genetic transformation and its high quality and ability to produce disease-free plants (Hartman *et al.*, 2004).

The main advantages of micropropagation of apple are:-

1. Enormous capacity to multiply target plant material compared to conventional cloning methods.
2. The ability to produce progeny all-year round.
3. The production of disease-free plant material.
4. The possibility of multiplying genotypes which produce seeds uneconomically or which are sterile (Dobranszki, and Teixeira da Silva, 2010).

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2.6 Methods of tissue culture

Two main methods of *in vitro* propagation can be distinguished:

1. Propagation from axillary or terminal buds, in which propagation is based on pre-existing meristems.

The following techniques are included:

a. Meristem-tip culture.

b. Shoot or shoot tip culture.

c. Node (single or multiple) culture.

2. Propagation by the formation of adventitious shoots or adventitious somatic embryos, based on explants originating from somatic tissues.

Adventitious shoot or embryo formation can occur:

a. Directly from tissues of the excised explants without previously formed callus (direct organogenesis or direct embryogenesis).

b. Indirectly, when shoots or embryos regenerate on previously formed callus or in cell culture (indirect organogenesis or indirect embryogenesis) (Dobranszki, and Teixeira da Silva, 2010).

2.7 Elimination of the browning

Cultivar Anna is very rich in phenolic compounds. This can be considered as handicap which hinders the success of the micropropagation technique. A protocol that could overcome tissues browning was found. The steeping of explants in antioxidants solutions AA and CA at 0.25% and 0.5% and the addition of the AC at 3.0 g/L in the culture medium is the most efficient solution (Boudabous *et al*., 2010).

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2.8 Stages of micropropagation

Generally, four stages are necessary for successful micropropagation (George and Debergh, 2008):

Stage 1- establishment of *in vitro* culture.

Stage 2- shoot multiplication.

Stage 3- rooting of microshoots.

Stage 4- acclimatization.

During Stage 1, explants were transferred to *in vitro* culture, which means that they could be surface sterilized so that they can survive and grow under artificial conditions. For successful initiation of an aseptic *in vitro* culture, stock plants could be selected and these or their parts often have to be pre-treated. Physical (e.g., light, temperature, etc.) or chemical pre-treatments can be necessary either for reducing the contamination of the stock plant in order to be able to have successful surface sterilization of explants or for enabling or improving the growth of explants in subsequent *in vitro* conditions. The process of stock plant selection and pre-treatments is defined as Stage 0 (George and Debergh, 2008).

After successful initiation of an aseptic culture an efficient and reliable method for shoot multiplication could be achieved during Stage 2. Its success depends on various factors, such as plant species, cultivar, or genotype; organic and inorganic compounds, plant growth regulator (PGR) content or consistency of the medium, and other physical culture conditions such as light, temperature, vessel humidity, etc. The aim of this stage involves inducing the development of *in vitro* shoots (microshoots) capable for further cycles of shoot multiplication (subcultures) or for introducing them into Stage 3 (rooting). During Stage 3 microshoots originating from Stage 2 should be rooted either under *in vitro* or under *ex vitro* conditions. Successful rooting depends on similar factors listed in Stage 2 (plant species, media and environment). The transfer of rooted shoots to the natural environment occurs during Stage 4 (Dobranszki and Teixeira da Silva, 2010).

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Rooting and successful acclimatization of regenerated apple shoots is necessary to obtain transformed plants. Although auxins were proved to be the most important factors in *in vitro* rooting process cytokinin content of proliferation media can also affect the rooting capacity of *in vitro* apple shoots (Magyar-Tabori *et al*., 2011).

2.9 Previous studies

2.9.1 Importance of hormones in apple tissue culture

Yepes & Aldwinckle, (1994) examined several factors that affect *in vitro* establishment, proliferation, and rooting of thirteen Malus cultivars and rootstocks. Apple shoot tips (1.5 ± 0.5 cm in length) were established using AA and CA as antioxidants. Four proliferation media containing 1.0 mgl^{-1} BAP and different concentrations of IBA and GA3 were tested. Proliferation rates varied depending on the genotype and medium used. The highest proliferation rate was obtained for a rootstock that produced 11.6 ± 2.5 shoots (1.5 ± 0.8 cm in length) per tube per month. Rooting was induced with IBA for all the genotypes tested. The optimal IBA concentration was cultivar dependent (between 0.1 and 1.0 mgl^{-1} IBA), and lower concentrations were necessary to induce rooting in liquid rather than in solid medium.

In 1999, Modgil *et al.* studied axillary buds of apple cultivar Tydeman's Early Worcester collected during the spring and summer seasons produced significantly higher percentage of explant establishment (75%) as compared to buds collected during other seasons. The buds were grown on MS mineral salts having 4.4 mM BAP, 2.8 mM GA3 and 0.5 mM IBA. Addition of different combinations and concentrations of antioxidants and adsorbents to the MS medium in the initial phase of culture reduced phenol exudation to some extent and increased survival percentage. The shoots were multiplied on MS medium with 2.2 mM BAP and 7.5 mM KIN (kinetin). For induction of roots, the shoots were kept in liquid medium (LM) containing 1.5 mM IBA and 15 g/L sucrose for 9 days before transferring them to an agar-solidified rooting medium. The micropropagated plants showed 90% survival in nursery conditions.

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Yancheva *et al.,* (2003) studied the effects of auxin and cytokinin on the regulation of apple cultivar Topred. Shoots development have been studied through shoots induction, differentiation and development. Leaves were cultured *in vitro* with TDZ (Thidiazuron) in combination with various auxins. Histological observations showed that within 3 days TDZ with 2,4-D (2,4-dichlorophenoxyacetic acid) enhanced more cell divisions than TDZ with IBA. One day of exposure to either TDZ_ IBA or TDZ_ 2,4-D, followed by culture on hormone free medium, initiated cell determination towards shoot regeneration but with only low rates of shoot formation (5_ 6%). Extension of the culture with TDZ_ IBA to 6 days, increased shoot regeneration to approximately 30% and treatment with TDZ_ IBA for the whole experimental period (35 days) increased it to 72%. However, a 2-day culture with TDZ_ 2,4-D, followed by TDZ_ IBA for 33 days increased regeneration to approximately 80%. TDZ with IBA or with IAA determined cell fate to shoot development, whereas TDZ_ 2,4-D application for more than 6 days led to the decline of shoot development. They conclude that in apple, the type of auxin, the timing of its application and the length of explant exposure to the specific auxin are critical for the activation and progression of the developmental program.

In 2005, Kaushal *et al*., studied *in vitro* clonal multiplication of apple rootstock M M 111 using axillary buds and shoot apices were carried out. Vegetative axillary buds of the size of 0.2-2.0 cm and shoot apices measuring 4 mm in length were initiated to shoot proliferation on MS medium supplemented with BA (0.5-1.0 mgl^{-1}) and GA_3(0.5 mgl^{-1}), with or without IBA (0.05-0.1 mgl^{-1}). Small size ex plants showed less phenol exudation and less contamination. Following establishment phase, the small shoots emerged from explants were subcultured on MS medium supplemented with different combinations and concentrations of growth regulators. BA (1.0 mgl^{-1}) and GA_3 (0.5 mgl^{-1}) combination showed highest multiplication rate (1:5), and also produced longer shoots. Two step rooting was done by transferring microcuttings to auxin free solid medium after root initiation in dark on 1/2 strength M S liquid medium containing IBA (0.5) and GA_3 (0.5 mgl^{-1}). Rooted plantlets were transferred

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to peat containing paper cups and resulting plants of MM 111 acclimated successfully for transfer to field.

In 2010, Boudabous,*et al*., studied a high frequency of sprouting (85.0%) and shoot differentiation was observed in the primary cultures of nodal explants of *Malus domestica* L. cultivar Douce de Djerba on MS medium supplemented with BAP (1.0 mgl^{-1}) plus IBA (0.1 mgl^{-1}). *In vitro* proliferated shoots are multiplied rapidly by culture of shoot tips on MS medium with BAP (1.0 and 2.0 mgl^{-1}) which produce the greatest multiple shoot formation. The BAP has a positive effect on the multiplication and growth, but a concentration that exceeds 4.0 mgl^{-1} decreases the growth. A high frequency of rooting (66.7%) with development of healthy roots is observed from shoots cultured on half strength MS medium enriched with IBA (3.0 mgl^{-1}) and 2.0 gl^{-1} of AC. Plants with well-developed roots were transferred to soil with a survival frequency of 60%.

Magyar-Tabori *et al*., (2011) studied rooting ability of '*Royal Gala'* shoots regenerated on media with different cytokinin was observed directly after regeneration and compared after subculture on hormone-free medium (A), or on medium with decreased cytokinin content (B), or elevated (GA3) content (C) for a week, or after a subculture on proliferation media for four weeks (treatment D). Rooting and acclimatization of regenerants after directly regeneration was not successful. Subculture of shoots on hormone-free medium did not improve the rooting ability of shoots, while their subculture on B and C media resulted in up to 36% rooting rate depending on regeneration media from which the shoots originated. The best rooting rate (up to 76%) was achieved in shoots regenerated on medium with treatment D similarly to rooting ability of micropropagated shoots (80%). The rooting capacity of shoots depended on both the cytokinin content of the regeneration media and different subculture media used. All rooted shoots survived after acclimatization.

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In 2011, Amiri and Elahinia studied the impact of medium composition PGRs (plant growth regulators) on multiplication rate, shoot elongation, callusing and rooting of apple rootstocks ('M9', 'M27', and 'MM106') cultured on gelled basal MS medium were investigated. Multiplication rate was mainly dependent upon kind of PGRs especially, BA, mineral concentration and genotypes. The best shoot production in terms of shoot number and shoot quality was obtained using 4.4 µM BA and 2.27 µM TDZ during the shoot multiplication phase, but 8.8 µM BAP + 1.14 µM TDZ and 2.8 µM (GA3) during the shoot elongation phase for all genotypes. Application of high (2.8 µM) concentration of GA3 increased the elongation of adventitious shoots than low concentrations. The highest multiplication rate (5.7 No./shoot) and the highest amount of total fresh weight (2.25 g/jar), as growth rate, were produced by applying 4.4 µM BA + 2.27 µM TDZ for 'M27' genotype. Micropropagation potential of 'M27' genotype was higher than other genotypes. 'MM106' genotype had the lowest multiplication rate (0.7 No./month), when 0 µM BAP+9.08 µM TDZ was applied. Multiplication of explants from the 1st subculture was more sensitive to BAP than that from the 3th or 4th subculture. The rooting of explants was promoted by (IBA) significantly and the best result for rooting was achieved in the half-strength MS medium containing 5.4 µM IBA and 1.2 µM (2,4-D). The highest percentage (64%) rooting was produced for 'MM106' genotype and the lowest (11%) for 'M9' after 3 months. Root formation was increased with decreasing concentrations in cytokinins, but increasing auxins (IBA). Rooting percentage of shoot cultures in the low 1/2X-MS medium was significantly more than shoot cultures in the high 2X-MS medium.

Keresa *et al*., (2012) examined the efficiency of axillary shoot proliferation on four media differing in PGRs and their concentrations. All media consisted of QL (Quoirin and Lepoivre) macroelements and (MS) microelements. Furthermore, rooting efficiency on six different media/treatments was analyzed. Media with1 mg/L BAP or 0.5 mg/L BAP + 1.5 mg/L Kin produced similar number of microshoots per inoculated one (2.5 and 2.4, respectively). Medium with 1 mg/L TDZ produced significantly higher number of shoots (3.6) but they were fasciated. Three different explant types also produced similar numbers of microshoots. High rooting efficiency

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(68.7%), a high number of roots per shoot (6.6) and the best quality of shoots were obtained in rooting medium containing 2 mg/L of IBA.

2.9.2 Importance of sucrose and Sodium chloride concentration on apple tissue culture

Calamar and Klerk, (2002) examined the effect of sucrose on adventitious root formation in apple microcuttings and in 1-mm stem slices cut from apple microcuttings. The sucrose concentration influenced the number of adventitious roots, but at a broad range of sucrose concentrations (1–9%) the effect was small. In addition, there was an interaction between sucrose and auxin: increasing the sucrose concentration shifted the dose–response curve of auxin to the right. When slices were cultured on medium without sucrose for the initial period (0–48 h), rooting was reduced whereas 48-h culture without application of sucrose had hardly any effect or even a slight promotive effect in a later period (48–120 h). The results show that during adventitious root formation, applied sucrose is used as a source of energy and building blocks but they are also in accordance with a possible regulatory role of sucrose.

Yaseen *et al*., (2009) studied the competence of two apple rootstocks M. 9 and M. 26 for *in vitro* shoot proliferation was appraised using a miscellany of carbon sources, sorbitol, sucrose, glucose and mannitol which were employed 0, 5, 15, 25, 35 and 45 g/L. The most auspicious outcome was achieved by sorbitol 35 g/L being the optimal carbon source for both the genotypes. M. 26 had a positive interaction with sorbitol at this concentration to produce the best caulogenic response in terms of a paramount shoot length (3.01 cm) and an overriding fresh weight increment (402 mg) whereas M. 9 at the same concentration gave an eminent shoot number. Sucrose and glucose also had a positive carryover effect on apple shoots to some extent but proved to be inferior to sorbitol. Results yielded by mannitol were highly indigent in comparison to other carbon sources. Rootstocks exhibited an inconsistency regarding their aptitude for shoot proliferation. M. 26 was recognized as a better rootstock with an

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acquisition of 1.05 cm shoot length and 154.6 mg fresh weight while M. 9 stood better with maximum shoot number of 2-3.

Bahmani *et al*., (2012) studied the *in vitro* response of MM.106 apple rootstock to increasing concentrations of NaCl (0, 20, 40, 80, 100 and 120 mM) in the MS culture medium was analyzed. Explant growth was seriously affected by salinity treatments. Elevated salinity from 20 (control) to 40, 80, 100 and 120 mM NaCl resulted in reduction in shoot growth (shoot number, length and fresh weight) and rooting (rooting percentage, root number and length). At 20 mM NaCl the shoot length, fresh weight and root length was increased significantly as compared with the control. At 120 mM NaCl the shoot length were adversely effected and only half length of that in the 20 mM NaCl, whereas the shoot number had slightly decreased.

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Chapter 3

Materials and Methods

All the laboratory activities and experiments were conducted in the Plant Tissue Culture Laboratory – Biology and Biotechnology Department at Islamic University-Gaza.

3.1 Materials

3.1.1 Chemicals

The chemicals listed in table (3.1).

Table 3.1 List of the chemicals used in this work

Chemicals	Manufactures
Composition of MS medium: **A. Macro Elements:** □ NH_4NO_3: 16.5 g/l □ KNO_3: 19 g/l □ $CaCl_2.2H_2O$: 4.4 g/l □ $MgSO_4.7H_2O$: 3.7 g/l □ KH_2PO_4: 1.7 g/l **B. Micro Elements:** □ Fe-Na-EDTA: 4 g/l □ H_3BO_3: 0.62 g/l □ $MnSO_4.4H_2O$: 2.23 g/l □ $ZnSO_4.7H_2O$: 0.86 g/l	Sigma Company, USA

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KI: 0.083 g/l $Na_2MoO_4.2H_2O$: 0.025 g/l $CuSO_4.5H_2O$: 0.0025 g/l CoCl2.6H2O: 0.0025 g/l	
Organic Constituents: Riboflavin Sucrose Agar	Hi Media Company, India
Hormones: (BAP) (IBA) (GA3)	Hi Media Company, India
Detergents: NaOCl Tween 20 $HgCl_2$ Ethyl alcohol 70%	Hi Media Company, India
Supplements: (AC)	Hi Media Company, India
Another: NaOH HCl	Sigma Company, USA

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3.1.2 Equipment

The main equipment listed in table (3.2).

Table 3.2 List of the main equipments used in this work

Instruments	**Manufactures**
Growth chamber	Made in Korea
PH meter	Made in Germany
Autoclave	Made in Germany
Micropipette	Made in Germany
Microwave	Made in Germany

3.2 Methods

3.2.1 Stock plant preparation

Mother plants of apple *Malus* × *domestica* Borkh. (cultivar Anna) grown in the farm land of Khan yonus city, Gaza Strip, were used as source of explants during this study. Shoot tips (1-2 cm in length) were taken from mother plants.

3.2.2 Stock solution preparation

(MS) basal medium used throughout this research activity. Initially, MS ready media from Sigma Company used in this study and full strength stock solutions of macronutrients, micronutrients and vitamins and other organic supplements separately prepared to use it in this research. The solution poured into plastic bottles and stored at 4° C until used. (PGRs) prepared in 1mg/ml concentration. The PGRs used for the study were the cytokinin, (BAP), the auxins, (IBA) or (IAA), and (GA3). The powdered crystal of the PGRs weighed and dissolved in distilled water to the required volume. Then they stored at a temperature of 4° C.

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3.2.3 Culture media preparation

In the first category of studies, the culture media for shoot initiation and multiplication contained full strength of MS basal medium, 30 g/l sucrose and with PGRs in the presence or absence of AC. In rooting experiments, half strength MS basal medium in the presence or absence of AC used with different concentrations of IBA or IAA for rooting. Seven gram per liter plant tissue culture agar used as a solidifying agent throughout the experiments. Finally, the pH of all media adjusted to 5.7 by using 1% HCl and/or NaOH after addition of agar and AC because the agar and AC used have shown slight increase in pH after addition to the media. After adjusting the pH, the gently mixed medium boiled in microwave until the agar melted. Then, 30 ml of the prepared medium dispensed into jars. The jars covered with caps immediately after dispensing the medium and autoclaved by steam sterilization at a temperature of 121°C and 10^5 KPa pressure for 15 minutes.

3.2.4 Surface Sterilization of Explants

Apical and axillary buds of Anna cut and collected by using strong blade. Explants rinsed under running tap water for 30 minutes. For surface sterilization, they treated with different concentrations of NaOCl solution 5% in pilot experiment, first experiment, second experiment, third experiment and the sixth experiment. We treated with 10% NaOCl solution in fourth experiment and 7% NaOCl in fifth experiment, this solution containing three drops of Tween 20 per 100ml for 20 minutes. Then plant materials rinsed five times with sterilized distilled water and treated with 75% alcohol for 30s followed by 0.5% $HgCl_2$ three drops of Tween-20 for 10 min. The explants were then thoroughly washed (4-5 washings) with sterilized distilled water to remove the traces of $HgCl_2$ (Dobranszki and Teixeira da Silva, 2010).

3.2.5 Establishment stage

After sterilization, explants shortened to remove the surfaces of explants and meristems trimmed to 3 mm as final explant in aseptic conditions and cultured on the

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already prepared culture initiation medium. All cultures in all experiments transferred and randomly placed on the growth room chamber with 16 hours photoperiod (8 hours dark) and 2700 lux light intensity at 25 ± 2º C. They transferred to new fresh medium every two weeks.

1. Pilot experiment

Initiation medium supplemented with (0.4 mg/l IBA, 0.1mg/l BAP and 0.2 mg/l GA3) in initiation medium (Naija *et al*., 2008). About 16 explants were used for this experiment.

2. First experiment

The experiment was repeated by using (0.4 mg/l IBA, 0.1mg/l BAP and 0.2 GA3).

in establishment medium (Naija *et al*., 2008). About 16 explants were used for this experiment.

3. Second experiment

Establishment medium supplemented with different concentrations hormones (BAP, IBA and GA3) as shown in (table 3.3). These media supplemented with 0.1 g/l AC (Dobranszki and Teixeira da Silva, 2010).

4. Third experiment

The experiments were repeated by using of hormones concentration within the group B only (B1, B2, B3 and B4) (Table 3.3) to get the new shoots. This media supplemented with 1 g/l AC (Wang *et al*., 1994). The solutions (AA and CA) 0.25% and 0.5%, respectively were the most efficient antioxidants (Boudabous *et al*., 2010).

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Table 3.3 Hormones concentrations in 10 groups which used in establishment stage

Groups \ Hormones		BAP (mg/L)	IBA (mg/L)	GA3 (mg/L)
A	A1	0.5	0.01	0.5
	A2	0.5	0.5	0.5
	A3	0.5	1	0.5
B	B1	0.5	0.1	0.5
	B2	1	0.1	0.5
	B3	1.5	0.1	0.5
	B4	2	0.1	0.5
C	C1	0.5	0.1	0.2
	C2	0.5	0.1	0.4
	C3	0.5	0.1	0.6

5. Fourth experiment

For establishment stage during this experiment, media were used without any additional hormones. This experiment was applied for establishment stage and kept in the dark for 10 days (Marks and Simpson, 1990). Twenty four jars were planted by this method. The explants were steeped in antioxidants solutions AA and CA at 0.25% and 0.5%. They cultured on MS medium with AC (1 g/l) .

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6. Fifth experiment

In this experiment, callus formation from buds was induced by using media contains BAP with 17.8µM and NAA (Naphthalene acetic acid) 2.7µM. Thirteen jars kept in dark for one month then transferred to the light in growth chambers (Wu *et al*., 2011).

7. Sixth experiment

Establishment medium supplemented with 0.1 mg/l IBA, 1.0 mg/l BAP and 0.5 mg/l GA3 (Dobranszki and Teixeira da Silva, 2010).

3.2.6 Shoot multiplication

1. Pilot experiment

All the samples contaminated in the establishment stage and they didn't reach to multiplication stage.

2. First experiment

All the samples oxidized in the establishment stage and they didn't reach to multiplication stage.

3. Second experiment

After growth on the initiation culture medium, young and healthy microshoots were cultured on shoot multiplication medium, full strength MS medium, containing different concentrations of PGRs table (3.4) (Yepes and Aldwinckle, 1994, Naija *et al*., 2008, Dobranszki and Teixeira da Silva 2010).

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Table 3.4 Hormones concentrations in 3 groups which used in multiplication stage

Hormones / Groups	BAP (mg/l)	IBA (mg/l)	GA3 (mg/l)
H1	1	0.1	0.5
H2	1	0	0.5
H3	0.4	0.1	0.2

We used five shoots per culture replications for each treatment. The culture jars labeled and randomly placed on the growth chambers with the same culture conditions (temperature, photoperiod and light intensity) as that of the initiation experiment. Sub-culturing made every two weeks to fresh medium of the same composition as the previous one. The growth response of the microshoots to different treatments carefully observed and recorded for four weeks.

4. Third experiment

4 young and healthy microshoots were cultured on shoot multiplication medium, containing 0.1 mg/l IBA, 1.0 mg/l BAP and 0.5 mg/l GA3 group H1(Table 3.4), and we used all other shoots in this experiment (directly to remain strong) in rooting stage.

5. Fourth, fifth, and sixth experiments

We didn’t reach for any suitable shoots from establishment stage.

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3.2.7 Rooting stage

1. Pilot experiment

We didn't reach for any suitable shoots from the end of establishment stage.

2. First experiment

All the shoots oxidized before reaching rooting stage.

3. Second experiment

The shoots treated with rooting media containing half strength MS basal media supplemented with different concentrations of IBA (0.5,1,2,3mg/l and 30mg/l for 3h) and without (AC) (Dobranszki and Teixeira da Silva 2010). We used 3 shoots after multiplication stage for each concentration.

4. Third experiment

A. *Ex vitro* rooting

Microshoots were rooted directly after dipping the bases of shoots into a powder containing 0.2% IBA and 10% Captan fungicide and planted the shoots in a seed tray with sterilized horticultural sand. The tray covered with a transparent lid and kept for 4 weeks at 22 °C (Dobranszki and Teixeira da Silva 2010).

B. *In vitro* rooting of microshoots

During this experiment five shoots which were taken after establishment stage were treated with rooting media, containing half strength MS basal media which prepared in the laboratory with omitting NH_4NO_3 from the rooting medium and supplemented with 2.66 μM riboflavin. This media was semi solid and contained half the amount of sugar (Puente and Marh, 1997).

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Four shoots which were taken after multiplication stage were treated with rooting media containing half strength MS basal media supplemented with 0.1 mg/l IAA and 1 mg/l IBA (Druart, 1997). The cultures were kept in dark for five days at a temperature of 25 ± 2º C. After five days, the cultures were transferred to the growth room chambers with 16 h photoperiod at 25 ± 2ºC. For those shoots grown in this medium, the shoots transferred to this medium after five days of growth in dark in the same medium composition. Physiological factors (photoperiod, temperature, and light intensity), pH of the medium, agar concentration and other growth conditions were the same as before. The numbers of roots produced from each shoot observed after four weeks of growth.

5. Fourth, fifth and sixth experiments

We didn't reach for any suitable shoots from the end of establishment stage, so we couldn't reach to rooting stage.

3.2.8 Experimental design and statistical data analysis

The number of surviving, oxidant and dead explants during culture establishment, number of buds, number of shoots and length of plant in different establishment and multiplication media, the number of roots per plantlet recorded and calculated. To detect the significance of differences among treatments at or below the probability level of 0.05, ANOVA (analysis of variance) was also made using the software.

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Chapter 4

Results

4.1 Surface Sterilization of Explants

The massive bacterial and fungal contamination at the initiation and multiplication stages was one of the main problems encountered with *in vitro* propagation. After transfer of the bud to solid sterile medium, a whitish exudate of bacteria and fungi observed around the base of the explants after 2-3 days. The problem complicated further by the latent nature of the contaminants. In general, it is accepted that contamination of plant tissue cultures can be caused mainly by insufficient aseptic techniques during manipulations, incomplete surface sterilization of the explants and endogenous microflora present in the explants. The most difficult stage in tissue culture is contamination and browning.

Explant browning due to oxidation of phenols and contamination were the two main problems associated with field-grown trees. However, cultures could be established at any time of the year, but success dependent on the time of year for collection of explants (Table 4.1).

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Table 4.1 Effect of surface sterilization treatments and seasons on contamination of *in vitro* bud culture of Anna (after one week)

The experiments	Sterilizing agents	Total No. of explants	contamination ratio	Oxidation Ratio	Healthy Ratio	Seasons of the year
Pilot experiment	NaOCl 5.0%+Tween-20	16	100%	0%	0%	Summer (Jul)
First experiment	NaOCl 5.0%+Tween-20+70%alcohol+0.5% $HgCl_2$	16	6.2%	18.8%	75%	Summer (Aug)
Second experiment	NaOCl 5.0%+Tween-20+70%alcohol+0.5% $HgCl_2$	82	13.4%	25.6%	61%	Autumn (Oct)
Third experiment	NaOCl 5.0%+Tween-20+ 70%alcohol+0.5% $HgCl_2$	65	55.3%	10.7%	34%	Winter (Des)
Fourth experiment	NaOCl 10.0%+Tween-20+70%alcohol+0.5% $HgCl_2$	24	20.8%	79.2%	0%	Winter (Jan)
Fifth experiment	NaOCl 7.0%+Tween-20+ 70%alcohol+0.5% $HgCl_2$	13	23%	69.3%	7.7%	Winter (Jan)
Sixth experiment	NaOCl 5.0%+Tween-20+ 70%alcohol+0.5% $HgCl_2$	20	10%	20%	70%	Spring (Mar)

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In the pilot experiment all the explants contaminated because the sterilization protocol used was not enough.

1. In first experiment sterilization protocol was recalibrated to add new sterile materials, and this was an appropriate protocol. Contamination ratio reached to 6.2% and this was a satisfactory result for the summer, where it was difficult to get a full sterilization to the presence of fungi inside the plant (Table 4.1).
2. In second experiment, the same sterilization protocol was applied to 82 sample during the autumn. It was found that the percentage of contaminated plants reached to 13.4%.
3. In third experiment, the same sterilization protocol was applied to 65 sample during the winter found that the percentage of contaminated plants up to 55.3%.
4. We used the same sterilization protocol in fourth experiment with changing the NaOCl concentration from 5% to 10% in an attempt to reduce contamination during the winter, but the result was not satisfactory due to the death of tissue cells by the higher NaOCl concentration. 24 samples were used and the ratio of contamination was 20.8%.
5. We used the same sterilization protocol with change the NaOCl concentration to 7% in the fifth experiment in winter to reduce contamination ratio and get healthy samples, but the oxidation ratio was 69.3%.
6. For the last experiment we used the previously protocol where NaOCl concentration 5% but during the spring, so we used 20 samples and the contamination ratio reached to 10% (Table 4.1).

4.2 Establishment stage

1. Pilot experiment

The ratio of contamination reached to 100% (Table 4.1).

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2. First experiment

Buds started to develop microleaves after one week of culturing. There were 16 explants and after one week the percentage of contaminated explants was 6.2%. The longest sample arrived to 2.2 cm and there had 7 shoots after 4 weeks.

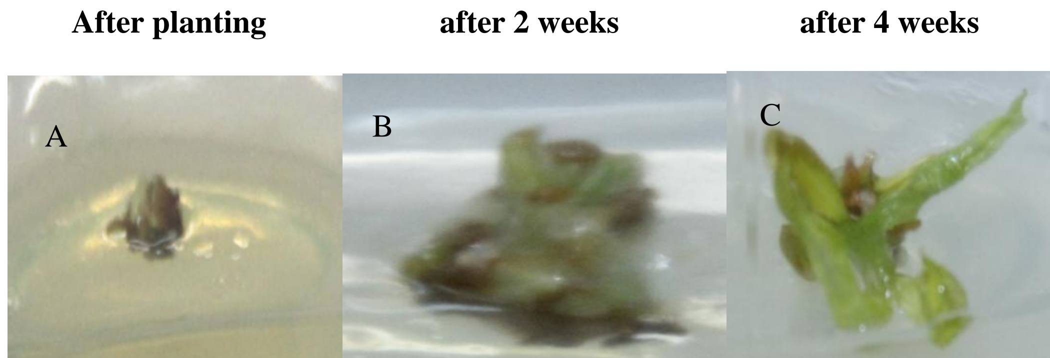

Figure 4.1 Establishment stage (A. after planting B. after 2 weeks C. after 4 weeks)

3. Second experiment

Buds started to develop microleaves after one week of culturing. Though they cultured on a medium with AC (0.1 g/l), the explants had brown color before producing microleaves. There were 82 explants and after one week the percentage of contaminated explants was 13.4%. After four weeks of growth on the initiation medium, 61% of Anna explants survived (Table 4.1) and responded better on MS medium containing 1.0 mg/l BAP, 0.1 mg/l IBA and 0.5 mg/l GA3 group B2 (Table 4.17). The worst result in group C1 when the concentrations were 0.5 mg/l BAP, 0.1 mg/l IBA and 0.2 mg/l GA3 (Table 4.25). In other media, the results of the growth of buds differentiated between the best and worst result. When we compared between A, B and C groups after four weeks in table (4.14) and figure (4.14), we found significance between A, B and C groups in the length of explant and number of shoots in favor of group B and there is no significance between A, B and C groups in the number of buds.

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Table 4.2 Statistical analysis for establishment stage in autumn after one week (group A)

		Group	No. of explants	Means	Standard Deviation	F Value	Significance
First week	Length of explant (cm)	A1	5	0.32	0.04	3.391	0.063
		A2	5	0.36	0.09		
		A3	5	0.46	0.21		
		T.G	15	0.38	0.12		
	Number of buds	A1	5	0.40	0.55	2.36	0.136
		A2	5	1.40	0.14		
		A3	5	1.80	1.30		
		T.G	15	1.20	1.15		
	Number of shoots	A1	5	0.00	0.00	5.44	0.021
		A2	5	0.00	0.00		
		A3	5	1.40	1.34		
		T.G	15	0.52	0.99		

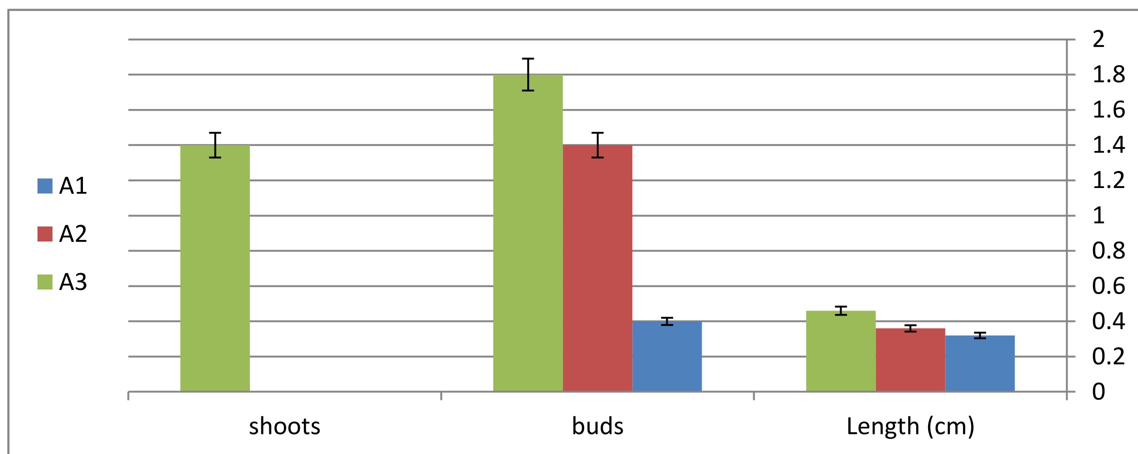

Figure 4.2 Establishment stage in autumn after one week (group A)

As shown in table (4.2) and figure (4.2), there is no significance between (A1, A2, A3) in length of explant and number of buds, while there is a significance between (A1, A2, A3) in number of shoots in favor of A3.

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Table 4.3 Statistical analysis for establishment stage in autumn after two weeks (group A)

		Group	No. of explants	Means	Standard Deviation	F Value	Significance
Second week	Length of explant (cm)	A1	5	0.36	0.55	3.39	0.068
		A2	5	0.44	0.15		
		A3	5	0.60	0.20		
		T.G	15	0.46	0.17		
	Number of buds	A1	5	0.40	0.55	2.52	0.125
		A2	5	2.00	1.73		
		A3	5	2.80	2.39		
		T.G	15	1.73	1.91		
	Number of shoots	A1	5	0.00	0.00	10.24	0.003
		A2	5	0.00	0.00		
		A3	5	2.60	1.82		
		T.G	15	0.86	1.62		

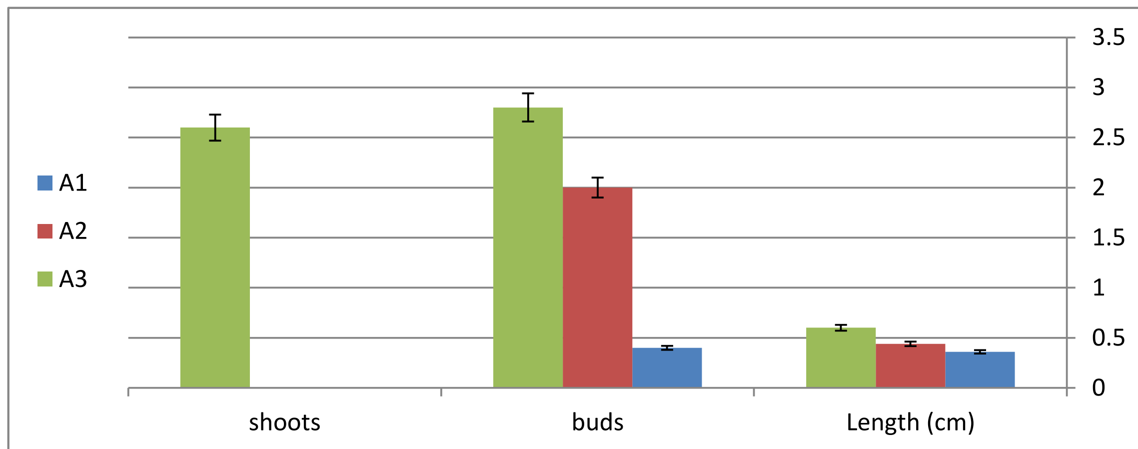

Figure 4.3 Establishment stage in autumn after two weeks (group A)

In the second week, table (4.3) and figure (4.3) also there is no significance between (A1, A2, A3) in length of explant and number of buds, while there is a significance between (A1, A2, A3) in number of shoots in favor of A3.

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Table 4.4 Statistical analysis for establishment stage in autumn after three weeks (group A)

		Group	No. of explants	Means	Standard Deviation	F Value	Significance
Third week	Length of explant (cm)	A1	5	0.46	0.11	2.28	0.144
		A2	5	0.66	0.31		
		A3	5	1.02	0.64		
		T.G	15	0.71	0.46		
	Number of buds	A1	5	0.40	0.55	4.44	0.036
		A2	5	3.60	2.88		
		A3	5	3.80	1.92		
		T.G	15	2.60	2.47		
	Number of shoots	A1	5	0.00	0.00	3.94	0.048
		A2	5	0.60	0.55		
		A3	5	3.20	3.27		
		T.G	15	1.26	2.28		

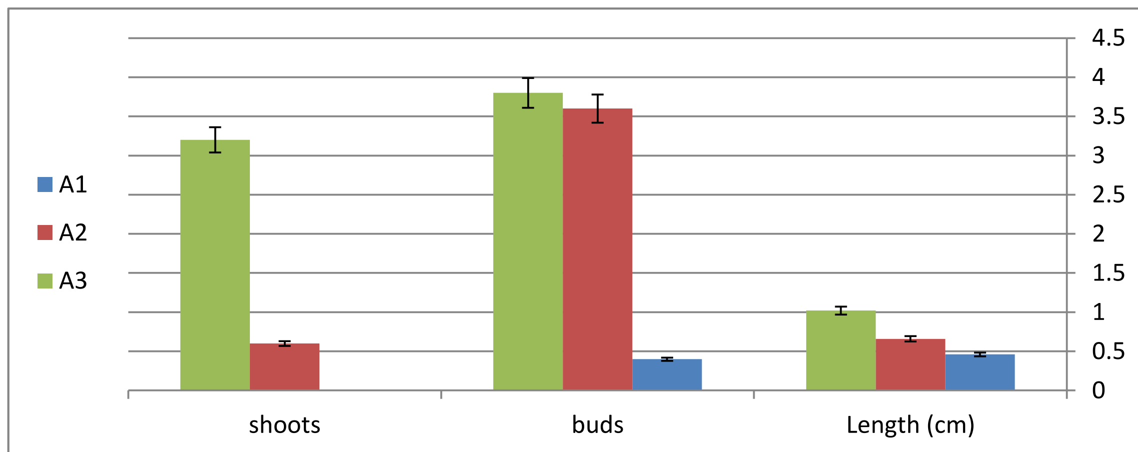

Figure 4.4 Establishment stage in autumn after three weeks (group A)

As shown in table (4.4) and figure (4.4), there is no significance between (A1, A2, A3) in length of explant, while there is a significance between (A1, A2, A3) in number of buds and number of shoots in favor of A3.

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Table 4.5 Statistical analysis for establishment stage in autumn after four weeks (group A)

		Group	No. of explants	Means	Standard Deviation	F Value	Significance
Fourth week	Length of explant (cm)	A1	5	0.50	0.12	4.26	0.040
		A2	5	1.02	0.45		
		A3	5	1.42	0.73		
		T.G	15	0.98	0.60		
	Number of buds	A1	5	0.60	0.55	3.64	0.058
		A2	5	3.40	4.39		
		A3	5	4.40	2.61		
		T.G	15	3.46	3.51		
	Number of shoots	A1	5	0.00	0.00	8.93	0.004
		A2	5	0.80	0.84		
		A3	5	3.60	2.30		
		T.G	15	1.46	2.11		

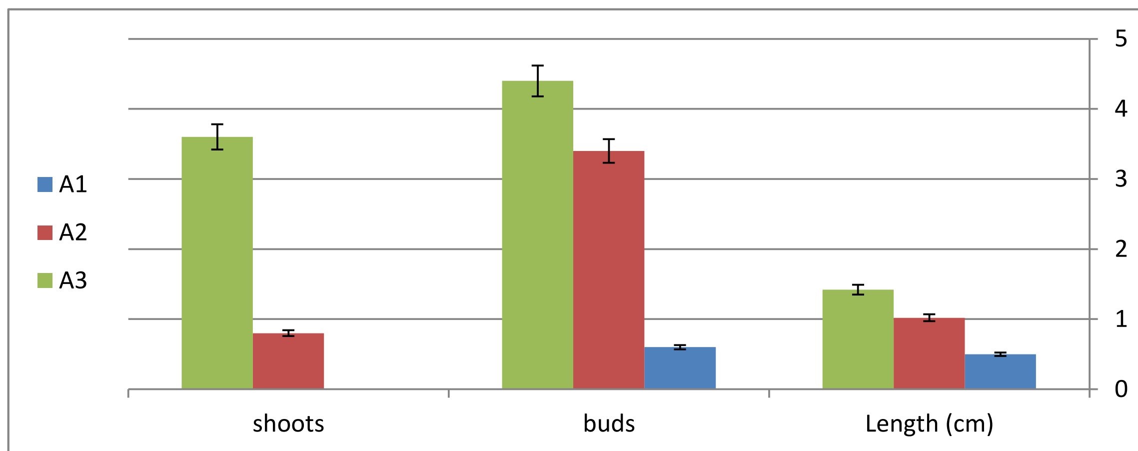

Figure 4.5 Establishment stage in autumn after four weeks (group A)

In table (4.5) and figure (4.5), there is a significance between (A1, A2, A3) in each of the length of explant, number of buds and number of shoots in favor of A3.

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Table 4.6 Statistical analysis for establishment stage in autumn after one week (group B)

		Group	No. of explants	Means	Standard Deviation	F Value	Significance
First week	Length of explant (cm)	B1	5	0.46	0.11	4.34	0.020
		B2	5	0.72	0.21		
		B3	5	0.60	0.20		
		B4	5	0.40	0.07		
		T.G	20	0.55	0.19		
	Number of buds	B1	5	2.80	1.30	1.82	0.184
		B2	5	4.00	2.34		
		B3	5	4.00	1.87		
		B4	5	1.80	1.30		
		T.G	20	3.15	1.87		
	Number of shoots	B1	5	0.20	0.44	5.95	0.006
		B2	5	2.40	1.14		
		B3	5	1.20	0.84		
		B4	5	0.80	0.84		
		T.G	20	1.15	1.14		

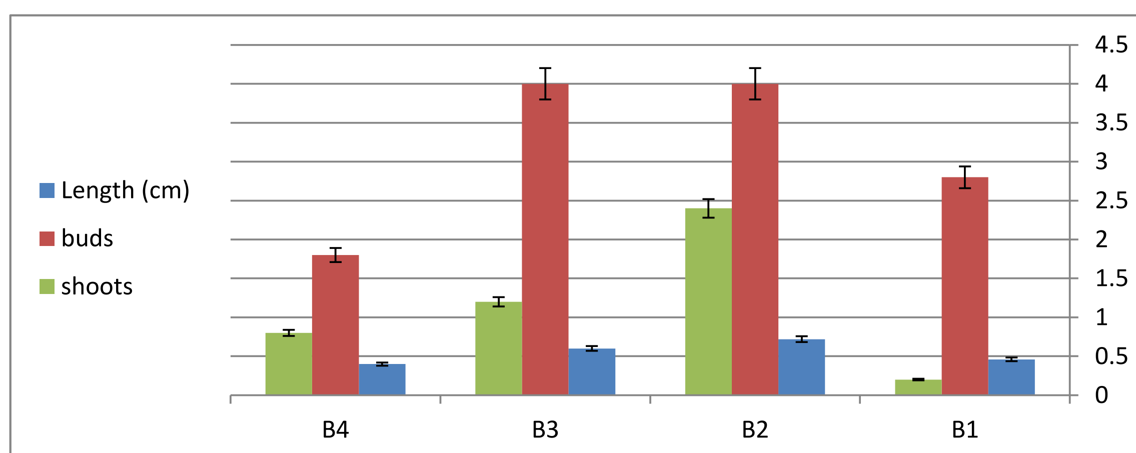

Figure 4.6 Establishment stage in autumn after one week (group B)

As shown in table (4.6) and figure (4.6), there is a significance between (B1, B2, B3, B4) in each of the length of explant, number of shoots in favor of B2, while there is no significance between (B1, B2, B3, B4) in number of buds.

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Table 4.7 Statistical analysis for establishment stage in autumn after two weeks (group B)

		Group	No. of explants	Means	Standard Deviation	F Value	Significance
Second week	Length of plant (cm)	B1	5	0.60	0.10	8.10	0.002
		B2	5	1.02	0.29		
		B3	5	0.84	0.21		
		B4	5	0.46	0.11		
		T.G	20	0.73	0.28		
	Number of buds	B1	5	3.80	1.30	0.70	0.564
		B2	5	4.20	1.92		
		B3	5	4.40	2.96		
		B4	5	2.60	2.07		
		T.G	20	3.75	2.11		
	Number of shoots	B1	5	0.40	0.54	9.00	0.001
		B2	5	3.80	1.11		
		B3	5	3.80	2.16		
		B4	5	1.20	0.84		
		T.G	20	2.30	1.97		

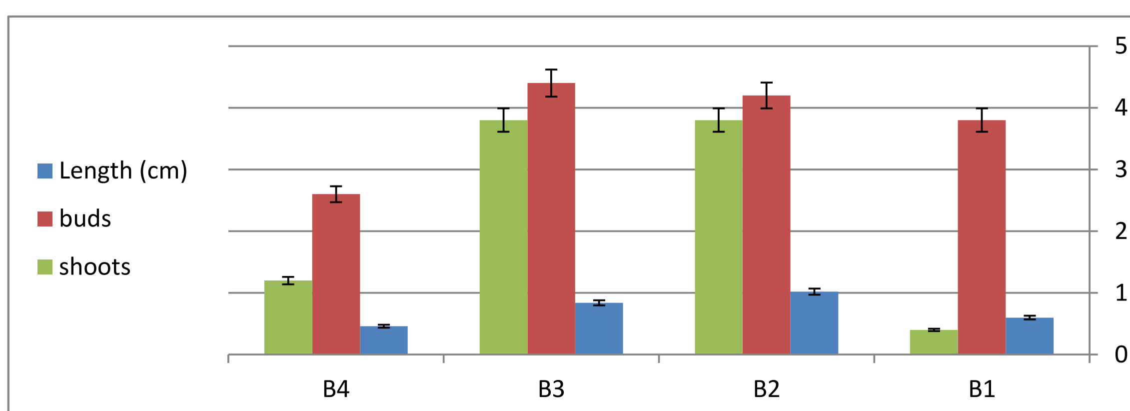

Figure 4.7 Establishment stage in autumn after two weeks (group B)

In the second week table (4.7) and figure (4.7), there is a significance between (B1, B2, B3, B4) in each of the length of explant for the benefit of B2 and in the number of shoots in favor of (B2 and B3), while there is no significance between (B1, B2, B3, B4) in number of buds.

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Table 4.8 Statistical analysis for establishment stage in autumn after three weeks (group B)

		Group	No. of explants	Means	Standard Deviation	F Value	Significance
Third week	Length of explant (cm)	B1	5	0.86	0.21	9.17	0.001
		B2	5	1.40	0.34		
		B3	5	1.04	0.21		
		B4	5	0.56	0.26		
		T.G	20	0.96	0.39		
	Number of buds	B1	5	2.40	1.67	2.91	0.066
		B2	5	2.20	2.38		
		B3	5	5.40	2.61		
		B4	5	2.20	0.84		
		T.G	20	3.55	2.28		
	Number of shoots	B1	5	2.80	1.11	2.56	0.091
		B2	5	5.20	1.48		
		B3	5	4.80	2.38		
		B4	5	2.80	1.92		
		T.G	20	3.90	1.99		

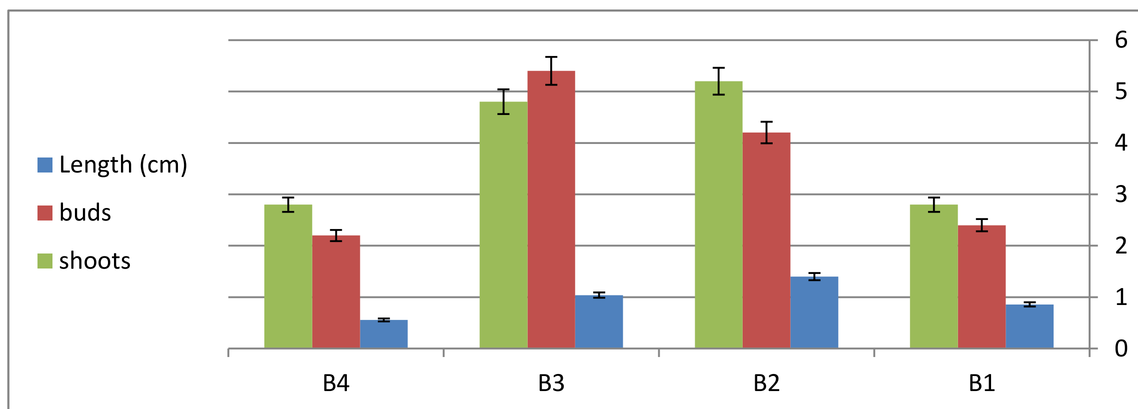

Figure 4.8 Establishment stage in autumn after three weeks (group B)

As shown in table (4.8) and figure (4.8), there is a significance between (B1, B2, B3, B4) in each of the length of explant for the benefit of B2, while there is no significance between (B1, B2, B3, B4) in number of shoots and number of buds.

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Table 4.9 Statistical analysis for establishment stage in autumn after four weeks (group B)

		Group	No. of explants	Means	Standard Deviation	F Value	Significance
Fourth week	Length of explant (cm)	B1	5	1.02	0.28	11.02	0.000
		B2	5	1.76	0.34		
		B3	5	1.38	0.22		
		B4	5	0.82	0.22		
		T.G	20	1.24	0.44		
	Number of buds	B1	5	3.40	2.11	1.44	0.268
		B2	5	5.00	2.55		
		B3	5	5.60	1.95		
		B4	5	3.60	1.14		
		T.G	20	4.40	2.10		
	Number of shoots	B1	5	2.80	1.30	5.14	0.011
		B2	5	6.40	1.14		
		B3	5	5.00	2.35		
		B4	5	3.60	1.14		
		T.G	20	4.45	2.01		

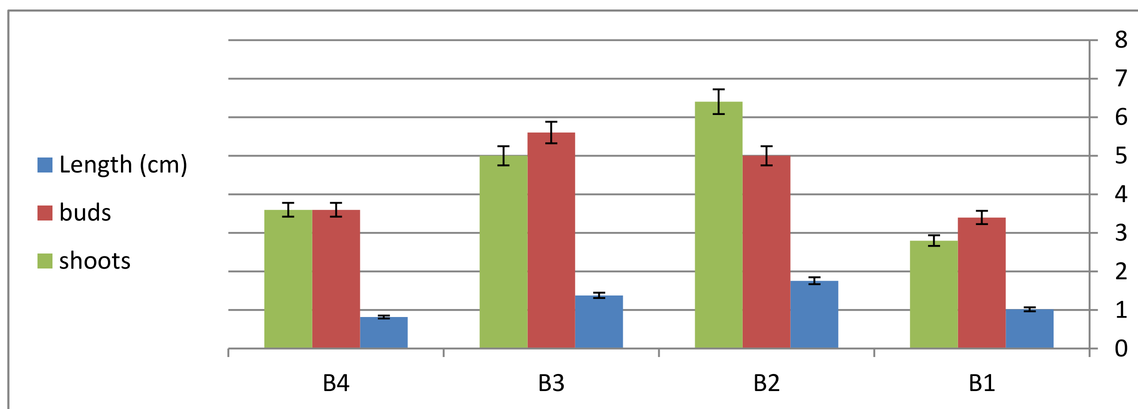

Figure 4.9 Establishment stage in autumn after four weeks (group B)

In table (4.9) and figure (4.9) there is a significance between (B1, B2, B3 and B4) in each of the length of explant and the number of shoots in favor of (B2). There is no significance between (B1, B2, B3 and B4) in number of buds.

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Table 4.10 Statistical analysis for establishment stage in autumn after one week (group C)

		Group	No. of explants	Means	Standard Deviation	F Value	Significance
First week	Length of explant (cm)	C1	5	0.34	0.05	0.22	0.800
		C2	5	0.34	0.05		
		C3	5	0.36	0.05		
		T.G	15	0.34	0.05		
	Number of buds	C1	5	0.60	0.54	2.59	0.116
		C2	5	2.00	1.22		
		C3	5	2.60	2.07		
		T.G	15	1.73	1.58		
	Number of shoots	C1	5	0.00	0.00	-	-
		C2	5	0.00	0.00		
		C3	5	0.00	0.00		
		T.G	15	0.00	0.00		

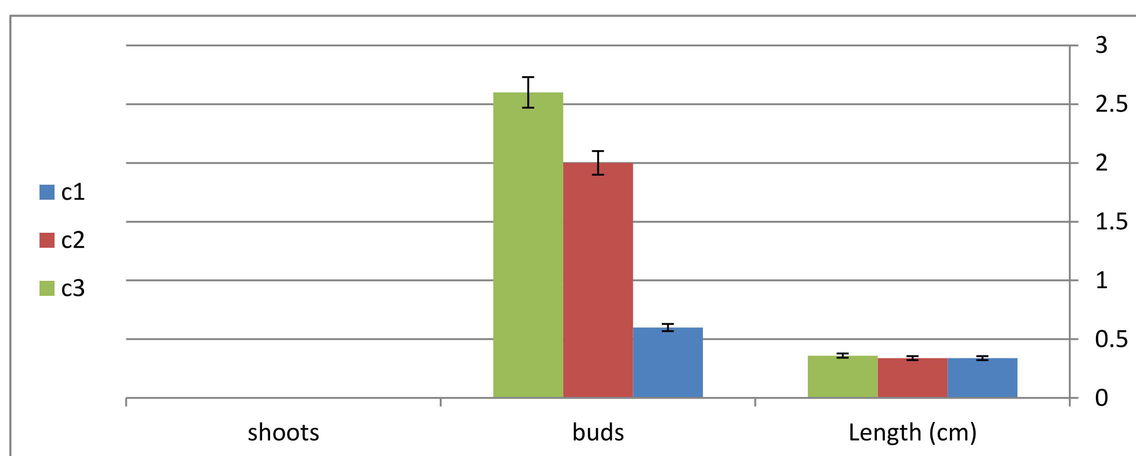

Figure 4.10 Establishment stage in autumn after one week (group C)

In the first week table (4.10) and figure (4.10), there is no significance between (C1, C2, C3) in length of explant, number of buds and number of shoots.

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Table 4.11 Statistical analysis for establishment stage in autumn after two weeks (group C)

		Group	No. of explants	Means	Standard Deviation	F Value	Significance
Second week	Length of explant (cm)	C1	5	0.36	0.05	1.00	0.397
		C2	5	0.36	0.09		
		C3	5	0.42	0.08		
		T.G	15	0.38	0.07		
	Number of buds	C1	5	1.40	0.55	2.13	0.161
		C2	5	2.80	2.05		
		C3	5	3.80	2.39		
		T.G	15	2.66	1.99		
	Number of shoots	C1	5	0.00	0.00	-	-
		C2	5	0.00	0.00		
		C3	5	0.00	0.00		
		T.G	15	0.00	0.00		

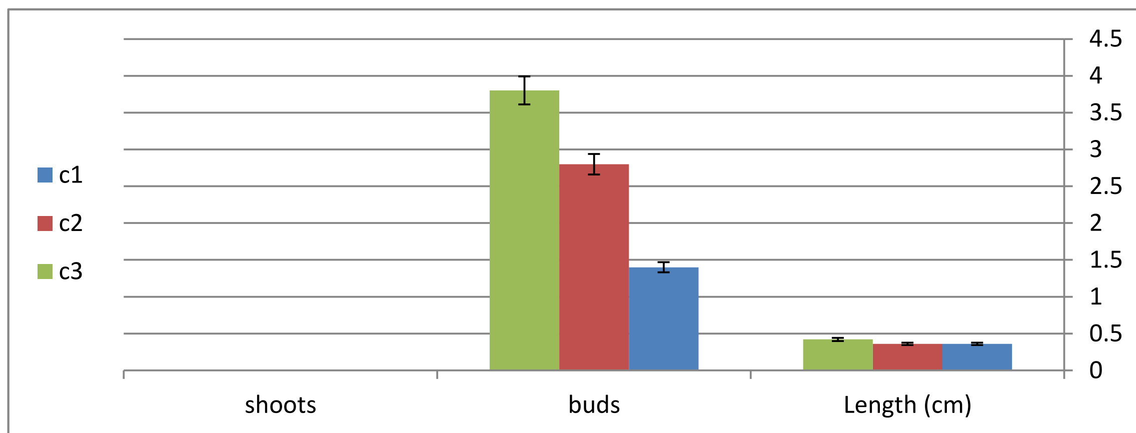

Figure 4.11 Establishment stage in autumn after two weeks (group C)

As shown in table (4.11) and figure (4.11) there is no significance between (C1, C2, C3) in length of explant, number of buds and number of shoots.

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Table 4.12 Statistical analysis for establishment stage in autumn after three weeks (group C)

		Group	No. of explants	Means	Standard Deviation	F Value	Significance
Third week	Length of explant (cm)	C1	5	0.36	0.05	5.77	0.018
		C2	5	0.40	0.07		
		C3	5	0.60	0.19		
		T.G	15	0.45	0.16		
	Number of buds	C1	5	1.60	0.54	4.07	0.045
		C2	5	3.20	1.92		
		C3	5	4.60	2.07		
		T.G	15	3.13	1.99		
	Number of shoots	C1	5	0.00	0.00	1.00	0.397
		C2	5	0.20	0.44		
		C3	5	0.00	0.00		
		T.G	15	0.07	0.26		

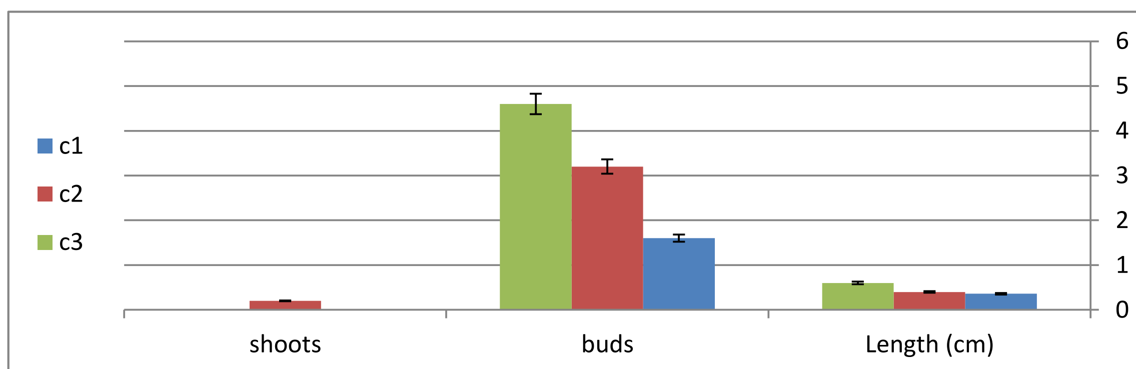

Figure 4.12 Establishment stage in autumn after three weeks (group C)

As shown in table (4.12) and figure (4.12), there is a significance between (C1, C2, C3) in the length of explant and number of buds in favor of C3 but there is no significance between (C1, C2, C3) in the number of shoots.

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Table 4.13 Statistical analysis for establishment stage in autumn after four weeks (group C)

		Group	No. of explants	Means	Standard Deviation	F Value	Significance
Fourth week	Length of explant (cm)	C1	5	0.42	0.04	4.23	0.041
		C2	5	0.54	0.09		
		C3	5	0.64	0.18		
		T.G	15	0.53	0.14		
	Number of buds	C1	5	1.60	0.54	3.96	0.048
		C2	5	3.20	2.28		
		C3	5	4.80	2.05		
		T.G	15	3.20	2.14		
	Number of shoots	C1	5	0.00	0.00	6.00	0.016
		C2	5	0.60	0.55		
		C3	5	0.00	0.00		
		T.G	15	0.20	0.41		

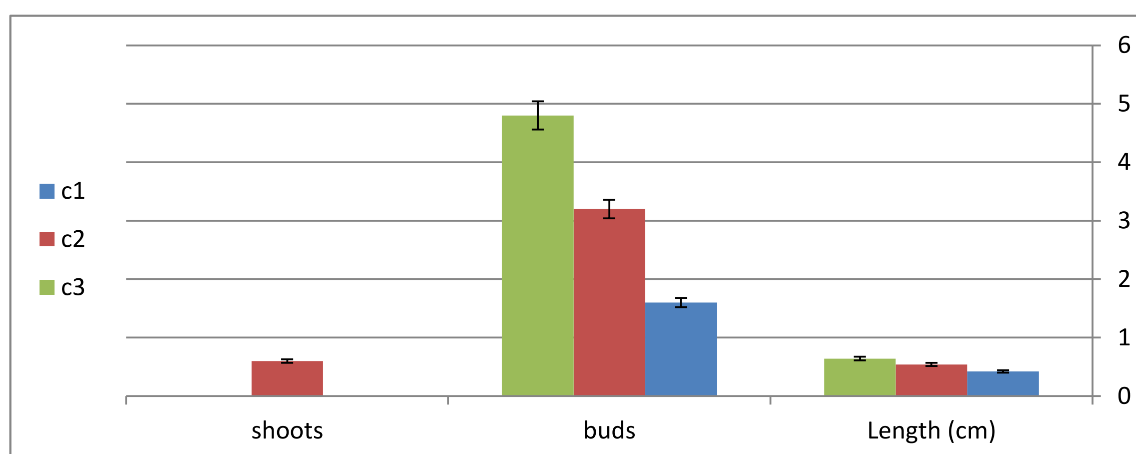

Figure 4.13 Establishment stage in autumn after four weeks (group C)

In table (4.13) and figure (4.13), there is a significance between (C1, C2, C3) in the length of explant and number of buds in favor of C3 and there is significance between (C1, C2, C3) in the number of shoots in favor of C2.

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Table 4.14 Statistical analysis for comparison between A, B and C groups in establishment stage after four weeks

		Group	No. of explants	Means	Standard Deviation	F Value	Significance
After four weeks	Length of explant (cm)	A B C	15 20 15	0.98 1.24 0.53	0.60 0.44 0.14	8.70	0.010
	Number of buds	A B C	15 20 15	3.46 4.40 3.20	3.51 2.10 2.14	1.13	0.371
	Number of shoots	A B C	15 20 15	1.46 4.45 0.20	2.11 2.01 0.41	6.48	0.020

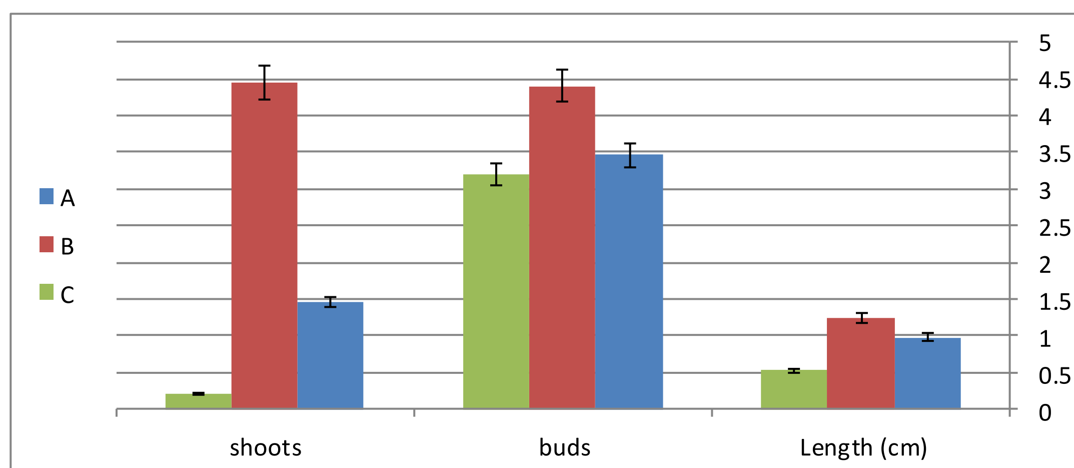

Figure 4.14 Comparison between A, B and C groups in establishment stage after four weeks

In table (4.14) and figure (4.14), there is a significance between A, B and C groups in the length of explant and number of shoots in favor of group B and there is no significance between A, B and C groups in the number of buds.

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4. Third experiment

Buds started to develop microleaves after one week of culturing. We cultured buds on a MS medium with AC (1 g/l), the explants had brown color with less amount before producing microleaves because of the usage of AA and CA as antioxidant. There were 65 explants and after one week the percentage of contaminated explants is 55.3%.

Table4.15 Statistical analysis for establishment stage in winter after one week (group B)

		Group	No. of explants	Means	Standard Deviation	F Value	Significance
First week	Length of explant (cm)	B1	5	0.50	0.16	2.36	0.110
		B2	5	0.80	0.31		
		B3	5	0.74	0.21		
		B4	5	0.70	0.70		
		T.G	20	0.68	0.21		
	Number of buds	B1	5	1.80	0.84	2.48	0.098
		B2	5	3.00	1.00		
		B3	5	2.20	0.84		
		B4	5	3.00	0.71		
		T.G	20	2.50	0.95		
	Number of shoots	B1	5	0.00	0.00	-	-
		B2	5	0.00	0.00		
		B3	5	0.00	0.00		
		B4	5	0.00	0.00		
		T.G	20	0.00	0.00		

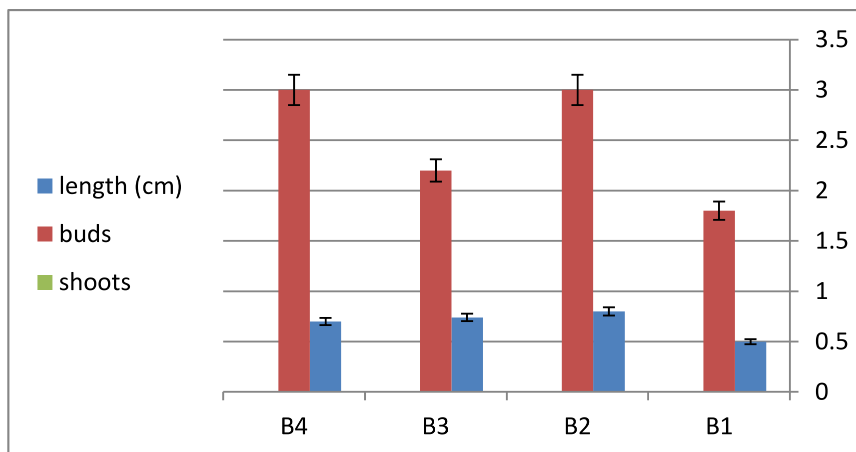

Figure 4.15 Establishment stage in winter after one week (group B)

In the first week table (4.15) and figure (4.15) there is no significance between (B1, B2, B3, B4) in each of the length of explant, number of shoots and number of buds.

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Table 4.16 Statistical analysis for establishment stage in winter after two weeks (group B)

		Group	No. of explants	Means	Standard Deviation	F Value	Significance
Second week	Length of explant (cm)	B1	5	0.82	0.25	0.80	0.511
		B2	5	1.00	0.35		
		B3	5	0.88	0.32		
		B4	5	0.76	0.05		
		T.G	20	0.86	0.25		
	Number of buds	B1	5	3.40	1.67	1.12	0.386
		B2	5	4.40	1.34		
		B3	5	3.20	1.30		
		B4	5	4.40	1.14		
		T.G	20	3.85	1.42		
	Number of shoots	B1	5	0.20	0.44	0.74	0.543
		B2	5	0.40	0.89		
		B3	5	0.60	0.89		
		B4	5	0.00	0.00		
		T.G	20	0.30	0.66		

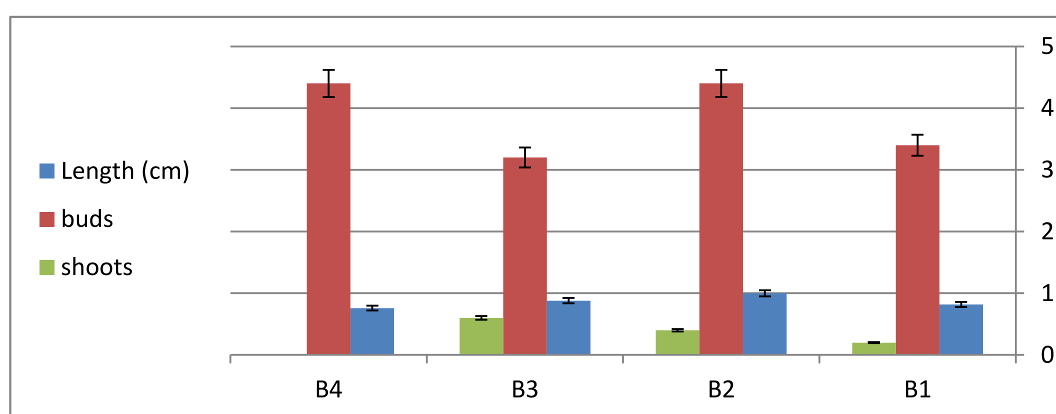

Figure 4.16 Establishment stage in winter after two weeks (group B)

Also in table (4.16) and figure (4.16) there is no significance between (B1, B2, B3, B4) in each of the length of explant, number of shoots and number of buds.

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Table 4.17 Statistical analysis for establishment stage in winter after three weeks (group B)

		Group	No. of explants	Means	Standard Deviation	F Value	Significance
Third week	Length of explant (cm)	B1	5	0.94	0.33	0.47	0.706
		B2	5	1.00	0.42		
		B3	5	0.96	0.23		
		B4	5	0.80	0.10		
		T.G	20	0.93	0.27		
	Number of buds	B1	5	1.60	1.52	1.09	0.383
		B2	5	1.80	0.84		
		B3	5	1.80	0.84		
		B4	5	2.80	1.30		
		T.G	20	2.00	1.22		
	Number of shoots	B1	5	2.20	1.92	0.34	0.793
		B2	5	3.00	1.87		
		B3	5	2.80	2.58		
		B4	5	1.80	1.92		
		T.G	20	2.45	1.99		

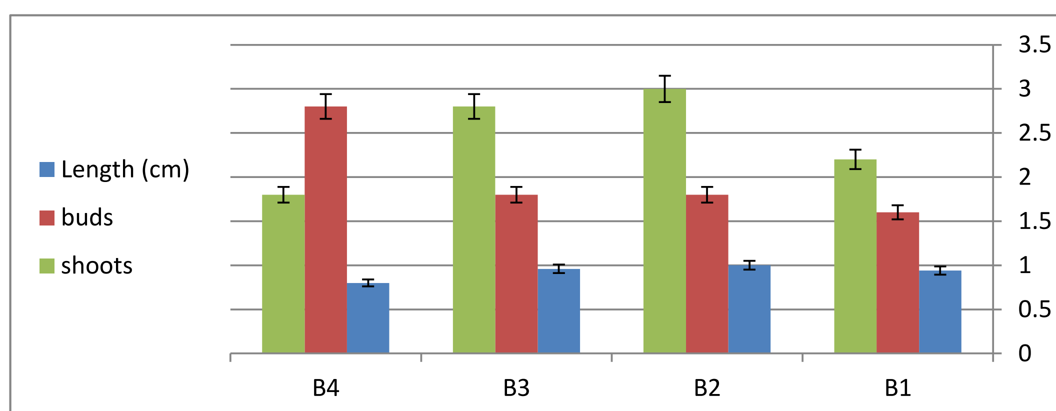

Figure 4.17 Establishment stage in winter after three weeks (group B)

In third week table (4.17) and figure (4.17) there is no significance between (B1, B2, B3, B4) in each of the length of explant, number of shoots and number of buds.

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Table 4.18 Statistical analysis for establishment stage in winter after four weeks (group B)

		Group	No. of explants	Means	Standard Deviation	F Value	Significance
Fourth week	Length of explant (cm)	B1	5	1.04	0.40	1.06	0.394
		B2	5	1.16	0.36		
		B3	5	1.02	0.26		
		B4	5	0.82	0.12		
		T.G	20	1.01	0.31		
	Number of buds	B1	5	3.00	1.41	0.19	0.899
		B2	5	2.40	1.67		
		B3	5	2.40	1.52		
		B4	5	2.80	1.48		
		T.G	20	2.65	1.42		
	Number of shoots	B1	5	2.60	2.30	0.37	0.773
		B2	5	3.60	1.34		
		B3	5	3.80	2.58		
		B4	5	3.20	1.12		
		T.G	20	3.30	1.84		

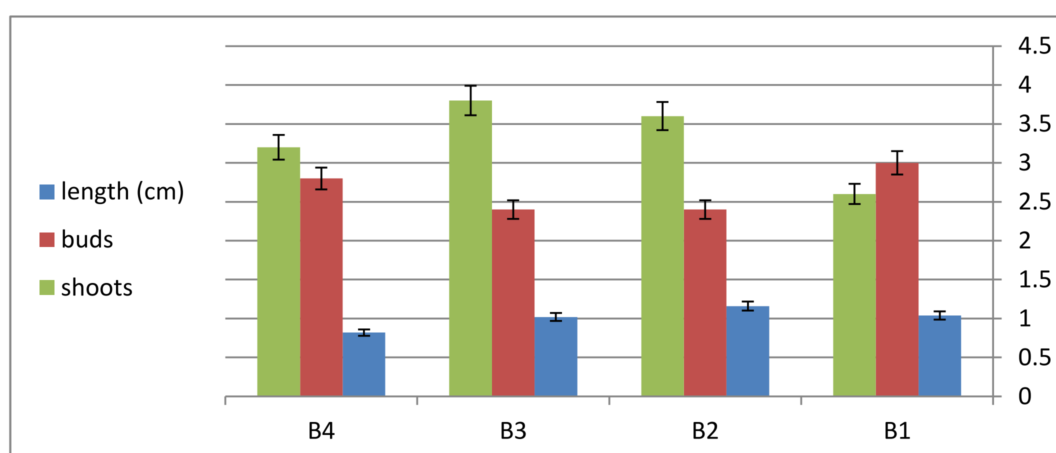

Figure 4.18 Establishment stage in winter after four weeks (group B)

As shown in table (4.18) and figure (4.18) there is no significance between (B1, B2, B3, B4) in each of the length of explant, number of shoots and number of buds.

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Table4.19 Statistical analysis for comparison between group B in Autumn and group B in Winter in establishment stage after four weeks

		Group	No. of explants	Means	Standard Deviation	F Value	Significance
After four weeks	Length of explant (cm)	B (Autumn) B (Winter)	20 20	1.24 1.01	0.44 0.31	2.03	0.171
	Number of buds	B (Autumn) B (Winter)	20 20	4.40 2.65	2.10 1.42	2.18	0.147
	Number of shoots	B (Autumn) B (Winter)	20 20	4.45 3.30	2.01 1.84	1.24	0.287

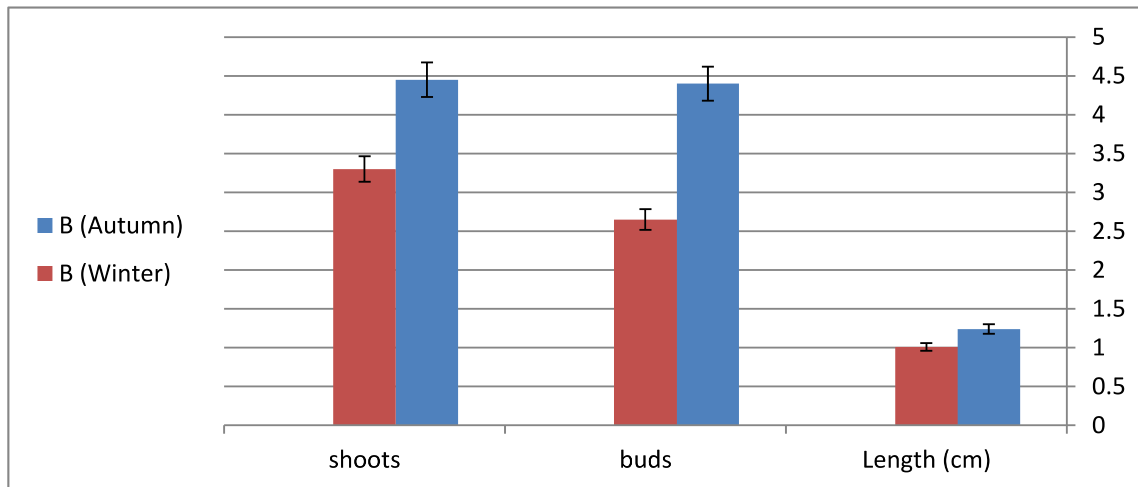

Figure 4.19 Comparison between group B in Autumn and group B in Winter in establishment stage after four weeks

As shown in table (4.19) and figure (4.19) there is no significance between group B in Autumn and group B in Winter in each of the length of explant, number of shoots and number of buds.

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5. Fourth experiment

Meristem tips cultured on hormone-free MS solid medium to establish clean and healthy materials for next experiments.

The ratio of pollution 20.8% and the rest of the samples did not succeed in growth due to the oxidation of the buds which was washed by using NaOCl 10%.

6. Fifth experiment (Callus stage)

13 samples were cultured for callus stage through segmentation buds. They were cultured on a medium without AC and kept in the dark. Pollution ratio was 23% and we had callus tissue only in a single sample, then callus tissue were oxidized during the subculture. We obtained two samples containing callus by using concentration B2, which in turn oxidized during the subculture.

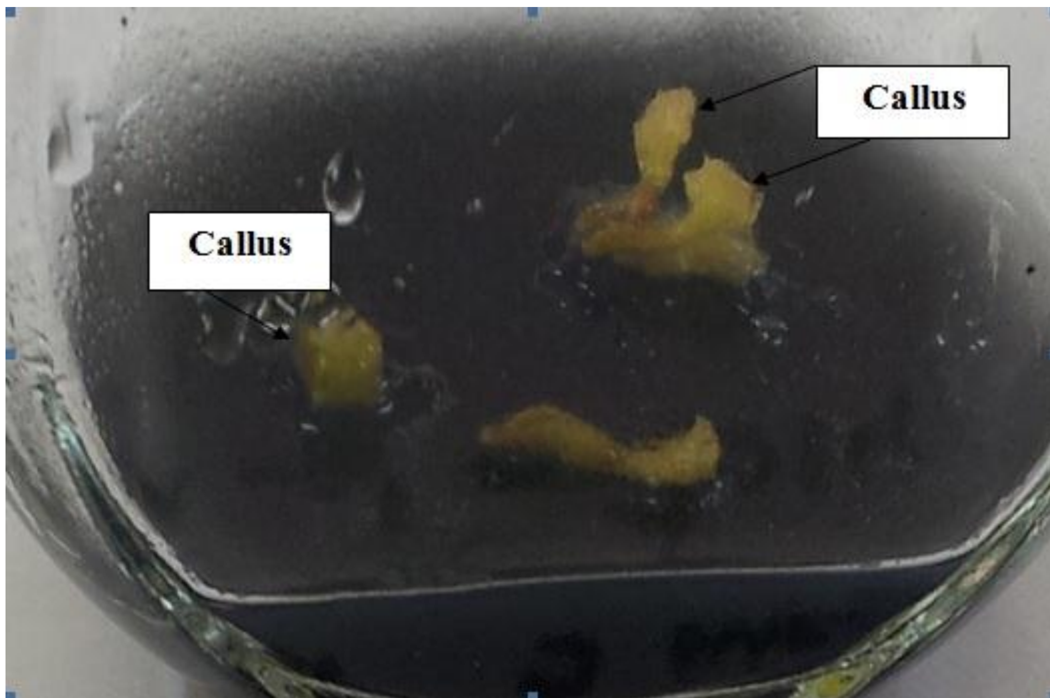

Figure 4.20 Callus inductions after 4 weeks in establishment media

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7. Sixth experiment

The ratio of contamination was 10% for the spring samples, the ratio of oxidation was 20% and the ratio of healthy was 70% (Table 4.1).

4.3 Shoot multiplication

1. Pilot experiment

In this experiment, it did not give any shoots for multiplication stage because sterilization protocol was not suitable.

2. First experiment

All samples oxidized in the establishment phase after the subculture and they did not reach to multiplication stage.

3. Second experiment

The shoots on multiplication medium gave different responses based on the different hormonal composition of the medium (Table 3.4). Only complete shoots obtained to their full propagation in H1 and H2 propagation media. Samples that treated with H3 media did not show any growth. This weak shoots, we enclosed them by using aluminum foil during the third week in order to retain their strength but there were some weak buds in some samples because of oxidation in these shoots.

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Table 4.20 Statistical analysis for multiplication stage after one week

		Group	No. of explants	Means	Standard Deviation	F Value	Significance
First week	Length of explant (cm)	H1	5	1.50	0.39	5.672	0.018
		H2	5	1.04	0.15		
		H3	5	1.02	0.15		
		T.G	15	1.19	0.33		
	Number of buds	H1	5	5.80	2.17	1.784	0.210
		H2	5	5.60	1.67		
		H3	5	3.80	1.64		
		T.G	15	5.06	1.94		
	Number of shoots	H1	5	3.40	0.89	0.034	0.967
		H2	5	3.60	1.67		
		H3	5	3.40	1.52		
		T.G	15	3.46	1.30		

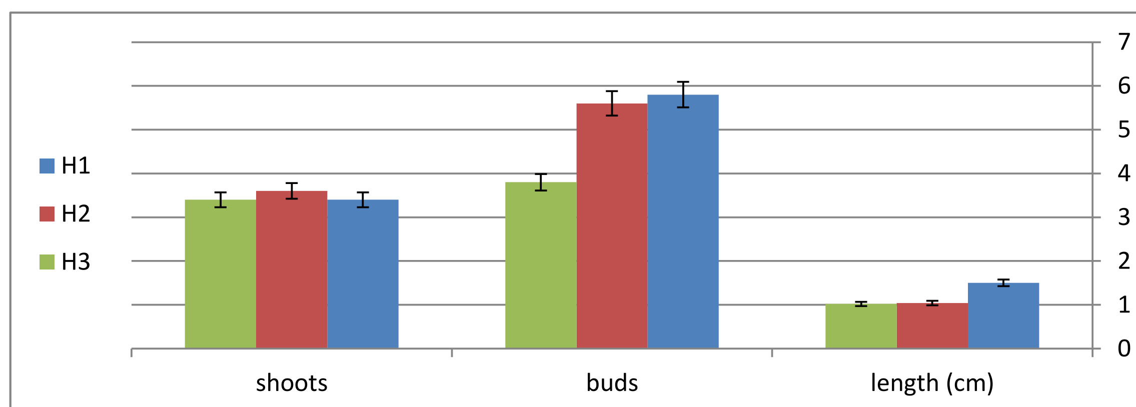

Figure 4.21 Multiplication stage after one week

As shown in table (4.20) and figure (4.21), there is a significance between (H1, H2, H3) in the length of explant in favor of H1, while there is no significance between (H1, H2, H3) in number of buds and number of shoots.

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Table 4.21 Statistical analysis for multiplication stage after two weeks

		Group	No. of explants	Means	Standard Deviation	F Value	Significance
Second week	Length of explant (cm)	H1	5	1.66	0.55	5.863	0.017
		H2	5	1.10	0.19		
		H3	5	0.96	0.13		
		T.G	15	1.24	0.44		
	Number of buds	H1	5	6.80	3.49	3.401	0.068
		H2	5	7.60	2.07		
		H3	5	3.80	1.09		
		T.G	15	6.06	2.81		
	Number of shoots	H1	5	5.00	1.41	1.680	0.227
		H2	5	4.40	1.95		
		H3	5	3.20	1.30		
		T.G	15	4.20	1.65		

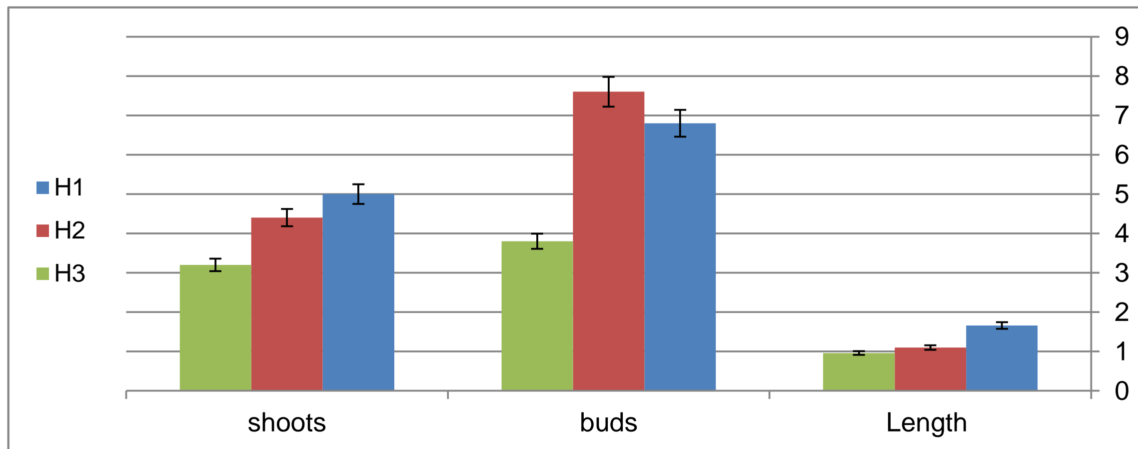

Figure 4.22 Multiplication stage after two week

As shown in table (4.21) and figure (4.22) there is a significance between (H1, H2, H3) in the length of explant in favor of H1 and number of buds in favor of H2, while there is no significance between (H1, H2, H3) in the number of shoots.

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Table 4.22 Statistical analysis for multiplication stage after three weeks

		Group	No. of explants	Means	Standard Deviation	F Value	Significance
Third week	Length of explant (cm)	H1	5	1.74	0.55	6.913	0.010
		H2	5	1.42	0.27		
		H3	5	0.90	0.10		
		T.G	15	1.35	0.49		
	Number of buds	H1	5	5.60	2.61	2.670	0.110
		H2	5	7.60	3.51		
		H3	5	3.80	1.09		
		T.G	15	5.66	2.89		
	Number of shoots	H1	5	6.40	3.36	2.994	0.088
		H2	5	5.00	1.87		
		H3	5	2.8.	1.30		
		T.G	15	4.73	2.65		

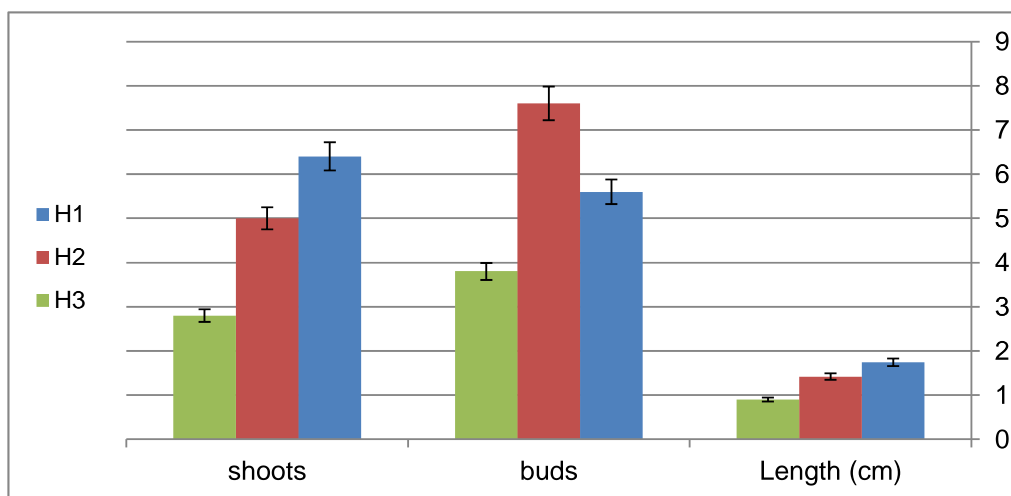

Figure 4.23 Multiplication stage after three weeks

In third week table (4.22) and figure (4.23) there is a significance between (H1, H2, H3) in the length of explant in favor of H1, while there is no significance between (H1, H2, H3) in number of buds and number of shoots.

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Table 4.23 Statistical analysis for multiplication stage after four weeks

		Group	No. of explants	Means	Standard Deviation	F Value	Significance
Fourth week	Length of explant (cm)	H1	5	1.88	0.62	8.395	0.005
		H2	5	1.58	0.35		
		H3	5	0.84	0.05		
		T.G	15	1.43	0.59		
	Number of buds	H1	5	6.80	3.27	4.599	0.033
		H2	5	7.80	2.05		
		H3	5	3.60	0.89		
		T.G	15	6.06	2.81		
	Number of shoots	H1	5	6.80	3.03	3.479	0.064
		H2	5	5.40	2.07		
		H3	5	3.20	0.84		
		T.G	15	5.13	2.53		

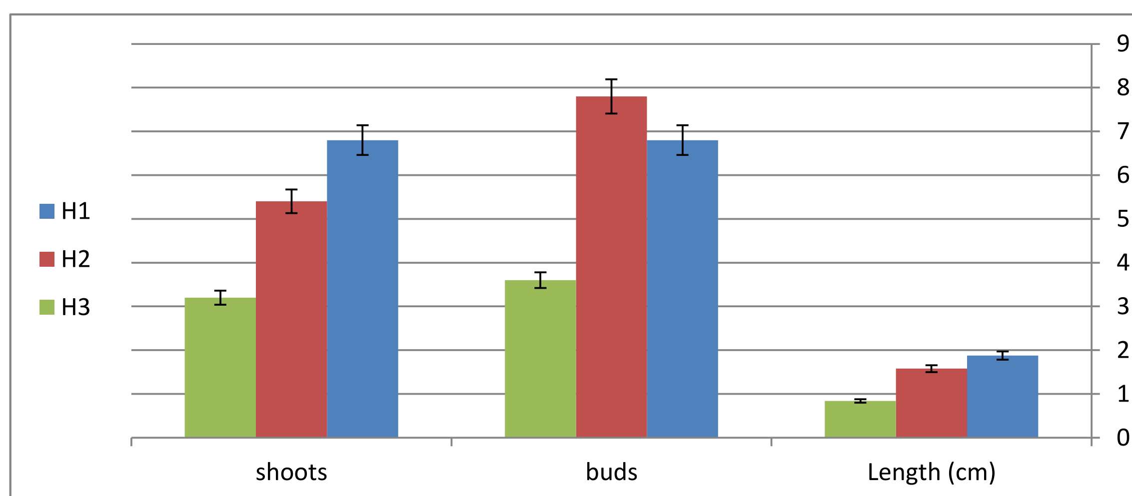

Figure 4.24 Multiplication stage after four weeks

As shown in table (4.23) and figure (4.24) there is a significance between (H1, H2, H3) in the length of explant in favor of H1 and number of buds in favor of H2.

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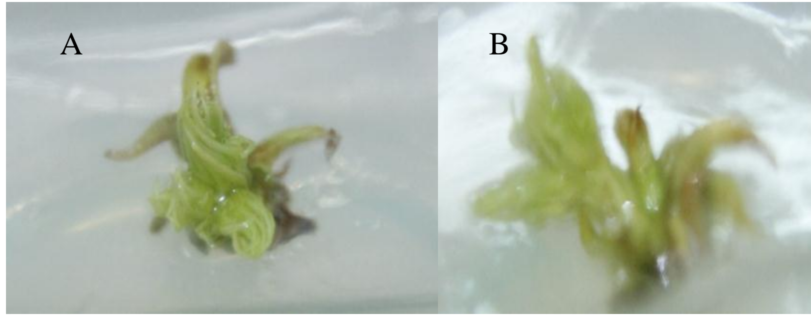


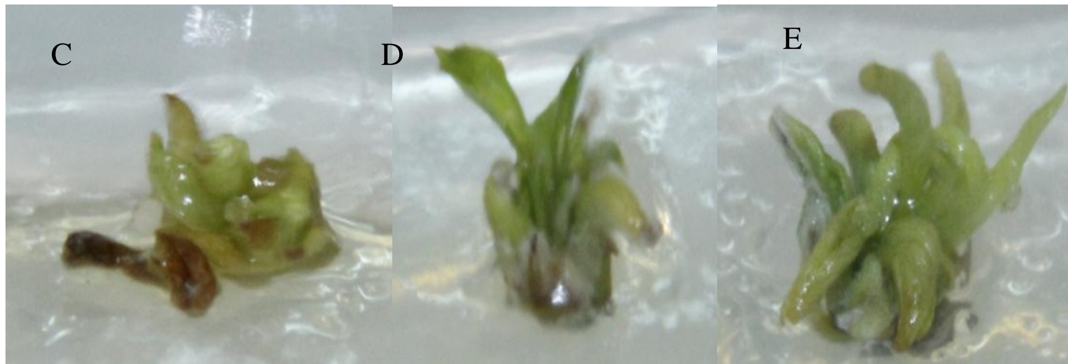
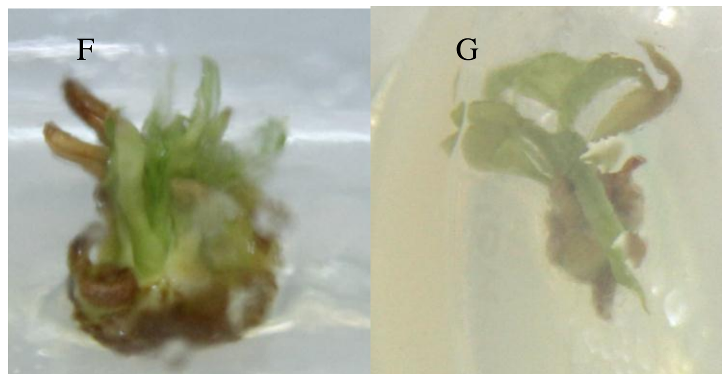


Figure 4.25 Multiplication stage in H1 media (A, B, C, D and E)



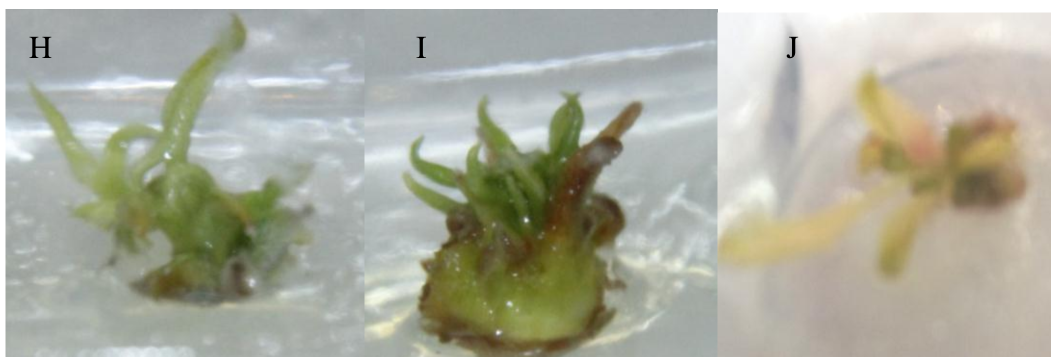


Figure 4.26 Multiplication stage in H2 media (F, G, H, I and J)

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4. Third experiment

4 microshoots were cultured on shoot multiplication medium, containing 0.1 mg/l IBA, 1.0 mg/l BAP and 0.5 mg/l GA3 group H1(Table 3.4), after multiplication stage we had young and healthy microshoots and there length were reached to 2.5 cm. We used all other shoots in this experiment after establishment stage (directly to remain strong) in rooting stage.

5. Fourth and fifth experiment

In these experiments, we hadn't any shoots for multiplication stage.

6. Sixth experiment

We stopped this experiment after one week because we did this experiment to calculate the healthy ratio (70%) of explants during spring.

4.4 Rooting stage

1. Pilot and first experiment

All the shoots were contaminated in the pilot experiment and were oxidized in the first experiment.

2. Second experiment

All the cultured shoots put in the dark for seven days, then transferred to auxin free media. All the cultured shoots of Anna didn't develop root on the rooting medium containing different concentrations of IBA (0.5, 1, 2, 3mg/l and 30mg/3h).

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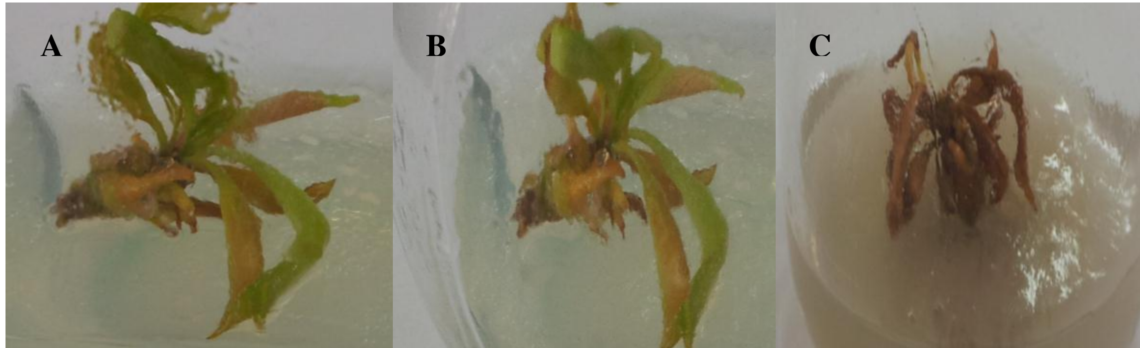

Figure 4.27 Rooting stage (A. after 5 days B. after 2weeks C. after 4 weeks)

2. Third experiment

1. *Ex vitro* rooting

We treated the shoots which were taken after establishment media (directly to remain strong) directly with dipping the bases of shoots into a powder containing 0.2% IBA and 10% Captan fungicide and planted the shoots in a seed tray with sterilized horticultural sand. The tray covered with transparent lid and kept for 4 weeks at 22 °C. All the cultured shoots of Anna didn't develop root on this rooting medium.

2. *In vitro* rooting of microshoots

A. We treated five shoots which were taken after establishment media (directly to remain strong) with rooting media containing half strength MS basal media, and we omitted NH_4NO_3 from this rooting medium and supplemented with 2.66 μM riboflavin. This media was semi solid and contained half the amount of sugar. All the cultured shoots of Anna didn't develop root on this rooting medium.

B. We treated four shoots which took after multiplication stage with rooting media containing half strength MS basal media supplemented with 0.1 mg/l IAA and 1 mg/l IBA. In the first sample, it formed 2 roots and root length arrived to

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2.1 cm. In the second sample, the number of roots formed was 3 and the length reached to 2.6 cm.

Figure 4.28 Rooting stage (after 4 weeks)

Then the samples subculture to a new media but these samples exposed to contamination, so we could not reach to the stage of acclimatization of this plant.

3. Fourth, fifth and sixth experiments

All of these experiments did not use in rooting stage. They did not give any shoots for rooting experiments.

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Chapter 5

Discussion

Tissue culture has been extensively used for raising multiple clones (micropropagation) of apple. Besides this, it is also useful in raising virus-free planting material, cryopreservation of genetic resources, development of synthetic seeds and apple improvement through transgenics (Dobranszki and Teixeira da Silva 2010). For micropropagation, explants are surface sterilized, inoculated on culture establishment/initiation medium, multiplied on the medium mostly consisting of cytokinins and are subjected to rooting. The success of micropropagation depends on various factors such as type of explant, season during its collection, age and genotype of stock plant, carbon source, composition of culture medium, (PGR), pH, culturing conditions, etc (Dobranszki and Teixeira da Silva 2010).

5.1 Effect of the season on the percentage of contamination of samples

Even though *in vitro* cultures can be established at any time of the year, early sprouting and maximum plant establishment observed with explants collected in spring or summer than those collected in other season (Modgil *et al*., 1999, Bhatti and Jha, 2010 and Mert and Soylu 2010). In this study a contamination rate of 6.2 and 10% observed when the explants collected during summer and spring, respectively, but the explants collected during autumn or winter showed a contamination rate of 13.8% or 55.3%, respectively (Table 4.1). High contamination ratio was 55.3% in winter because the fungi migrated inside the buds in winter to escape from the cold. These results are generally agreed with the previous results (Modgil *et al*., 1999, Bhatti and Jha, 2010 and Mert and Soylu 2010). The rate of uncontaminated/contaminated explants depends very strongly also on the different phytosanitary stage of the donor (Laimer da Camara Machado et al., 1991).

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5.2 Surface Sterilization of Explants

Different methods described for surface sterilization of explants. Our results showed the best protocol for surface sterilization of explants was in the first experiment in summer, this result agreed with (Modgil *et al*., 1999). It was an appropriate protocol, that's where contamination ratio reached to 6.2% and it was a satisfactory result. Our result agreed with (Wang *et al*. 1994 and Kaushal *et al*., 2005). It is difficult to get a full sterilization because the presence of fungi inside the plant (Dobranszki and Teixeira da Silva 2010). Our results showed that the highest contamination rates were in the third experiment in the winter 55.3% and this result agreed with previous study (Modgil *et al*., 1999).

5.3 Phenolic browning

Explant browning and eventual death of the tissue during the initial stage of apple culture is a frequent problem. It occurs through the action of PPO (polyphenol oxidase) and POX (peroxidases) by triggering defense reactions induced by wounding (Dobranszki and Teixeira da Silva 2010). PPO catalyzes the reaction between different phenolic compounds and molecular oxygen producing quinones which are highly reactive and non-specifically polymerize proteins and produce dark pigments, melanin (Onay and Jeffree, (2000), Leng *et al*., (2009)). In intact cells the enzymes and their substrates do not meet, because polyphenols, the substrates of PPO, are in the vacuoles, while the enzyme is in plastids or chloroplasts. When cells are wounded during explant excision, the browning reaction starts (Murata *et al*., 1997). The activity of enzymes associated with phenol oxidation is also affected by environmental factors. Light suggested to increase enzyme activity (Linington, 1991), while lowering temperature decreased phenolic biosynthesis by decreasing enzyme activity (Wang *et al*., 1994). We used explants 0.3 cm in size and this size agreed with (Kausal *et al*. 2005) who studied the effect of explant size. They concluded that axillary buds ranging from 0.2 to 0.6 cm in size showed less browning intensity and higher survival (60%) compared to larger (0.6–1 and 1–2 cm). Our results showed the less browning explants which collected in spring 20% or in summer 18.8%, this results agree with (Modgil *et al*., 1999), but the steeping of

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explants in antioxidants solutions AA and CA at 0.25% and 0.5% is the most efficient solution (Boudabous *et al*., 2010), so in the third experiment we got lower rates of oxidation 10.7% (Table 4.1).

5.4 Establishment stage

From this study, it was observed that, cultured buds gave varying responses to all of the culture media compositions.

1. Pilot experiment

Our results indicated that the contamination rates were 100% (Table 4.1) because sterilization method was not suitable and this result not agreed with previous study (Kausal *et al*., 2005).

2. First experiment

We obtained successful experiment on the growth of suitable shoots and the length of the samples reached to 2.2 cm. This corresponds to the study of (Naija *et al.,* 2008).

3. Second experiment

We obtained successful experiments on the growth of suitable shoots and the length of the samples reached to 2.2 cm in group B2. Our results in group A showed that there was a significance between A1,A2 and A3 in each of the length of explant and number of shoots in favor of A3 and number of buds in favor of A3 (Table 4.5 and figure 4.5). These results agreed with previous research (Dobranszki and Teixeira da Silva 2010). The best initiation medium supplemented with 1.0 mg/l BAP, 0.1 mg/l IBA and 0.5 mg/l GA3 (B2). These initiated shoots were having healthy morphology and had sufficient number of leaves and shoot height which facilitates their growth. This result was strongly agreed with previous reports (Sharma *et al.* 2000). Statistical analysis of group B showed that there was a significance between B1,B2,B3 and B4 in each of the length of explant and number of shoots in favor of B2 (table 4.9 and

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figure 4.9). Moreover, transfer of the cultured shoots to new fresh medium was important to reduce the risk of death of explants. This result was also in consistent with the previous work (Dalal *et al*., 2006). The worst initiation medium supplemented with 0.5 mg/l BAP, 0.1 mg/l IBA and 0.2 mg/l GA3 (C1). This result disagreed with (Dobranszki and Teixeira da Silva 2010). Statistical analysis in group C explained that there was a significance between C1,C2 and C3 in each of the length of explant and number of buds in favor of C3 and number of shoots in favor of C2 (Table 4.13 and figure 4.13). In table (4.14) and figure (4.14), there was a significance between A, B and C groups in the length of explant and number of shoots in favor of group B so in the third experiment we used the hormonal concentrations in group B.

4. Third experiment

In this experiment we tried to obtain the largest possible number of shoots, to be used in the process of rooting. Our results showed the usage of 1 mg\l AC enough for initiation media. The steeping of explants in winter in antioxidants solutions (AA and CA) at 0.25% and 0.5% was the most efficient solution (Boudabous *et al.,* 2010) so in the Third experament we got lower rates of oxidation 10.7%. This impacted on the growth of shoots in four groups (B1, B2, B3, and B4) so that there was no significant difference between them. This result agreed with the study of (Dobranszki and Teixeira da Silva 2010).

5. Fourth experiment

In this experiment, we used a high concentration of NaOCl to reduce the contamination ratio in the winter, but we did not get any healthy shoots after 4 weeks because the high concentration of NaOCl killed the plant tissue, and this is not consistent with the previous study (Grant and Hammatt, 1999).

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6. Fifth experiment

We optioned callus tissue just in one sample; these results disagreed with (Wu *et al.*, 2011). We thought that the high concentration of NaOCl which used 7% in sterilization process could have a negative effect on the health of tissues.

7. Sixth experiment

This experiment were worked in the spring to determine the percentage of contamination, oxidization and healthy ratio during this season and the result of ratio contamination was 10% (Table 4.1). This result agreed with (Modgil *et al.,* 1999).

5.5 Shoot multiplication

The success of shoot multiplication depends not only on the genotype, but also on PGRs and the interactions between these two factors. Shoot multiplication of apple is based on media containing cytokinins as the major PGR, and with lower concentrations of auxin and sometimes gibberellin (Dobranszky and Teixeira da Silva, 2010).

1. Pilot experiment

Our results indicated that the contamination rates were 100% in establishment stage (Table 4.1) because the sterilization protocol was not suitable, so we did not reach to multiplication stage in this experiment.

2. First experiment

Our results showed that all samples oxidized in the establishment phase after the subculture and they did not reach to multiplication stage because late in the subculture of samples to the stage of multiplication and it was compatible with the study of (Naija *et al.*, 2008).

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3. Second experiment

In the present study, full strength MS medium containing different types and concentrations of hormones used to assess the multiplication potential of the already initiated shoots of Anna apple variety. Our results showed in H1,H2 and H3 (Table 4.23), the maximum numbers of shoots for this variety counted in group H1 hormonal compositions, this result agreed with (Yepes and Aldwinckle, 1994). BAP is the preferred cytokinin for apple shoot multiplication; however, other analogs of benzyladenine could also enhance shoot proliferation (Dobranszki and Teixeira da Silva 2010). In line with this, (Hartman *et al*., 2004) suggested that, auxins should be either completely absent, or used in small concentration in shoot multiplication medium. There was weak multiplication of the plant by using H3 media and this result was inconsistent with the study of (Naija *et al.,* 2008). In table (4.23) and figure (4.24) statistical analysis in group H explained that there was a significance between H1, H2 and H3 in each of the length of explant and number of shoots in favor of H1 and number of buds in favor of H2.

4. Third experiment

Our results showed suitable shoots in H1 multiplication medium, this result agreed with (Yepes and Aldwinckle, 1994). We used other strong shoots directly in rooting stage to increase success in rooting stage.

5. Fourth experiment

In this experiment buds did not give any shoots for multiplication stage, because the high concentration of NaOCl killed the plant tissue, and this was inconsistent with the study of (Grant and Hammatt, 1999).

6. Fifth experiment

We aimed to get callus tissue in this experiment, but all the samples oxidized through establishment stage after 4 weeks, so we did not get any shoots for multiplication stage.

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7. Sixth experiment

Our results showed that this experiment worked in the spring to determine the percentage of contamination during this season and the result of ratio contamination was 10%. This result agreed with (Modgil *et al*., 1999, Bhatti and Jha, 2010 and Mert and Soylu 2010) who studied effect the season on ratio of contamination during establishment stage, so the spring season came in the second place after the summer in the establishment stage for the micropropagation of apple buds.

5.6 Rooting stage

1. Pilot experiment

In this experiment, it did not reach to rooting stage because all the samples contaminated in establishment stage.

2. First experiment

Here, also we did not reach to rooting stage because all the shoots oxidized in establishment stage because the late in the subculture of shoots to the multiplication stage or rooting stage.

3. Second experiment

We assessed the rooting response of Anna apple variety; Dalal *et al*., (2006) and Sharma *et al.,* (2007) found that IBA was the most effective for root induction *in vitro*. Half MS medium was recommended by different authors for rooting *in vitro* (Hartman *et al.,* 2004, Dalal *et al.,* 2006 and Bahmani *et al.,* 2009). Half MS medium containing IBA at different concentrations was used, but our results showed that we did not get any roots because exposure Growth Champers to malfunction and overheating several times, these results were inconsistent with the (Dalal *et al.,* 2006; Sharma *et al.,* 2007).

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4. Third experiment

A. *Ex vitro vs. in vitro* rooting

Our results show that there were no roots formed when we used sterilized horticultural sand for *in vitro* rooting. So this result disagreed with study of (Dobranszki and Teixeira da Silva 2010).

B. *In vitro* rooting of microshoots

1- All the cultured shoots of Anna didn't develop root on the rooting medium in this experiment, this result disagreed with (Puente and Marh, 1997).

2- We treated four shoots of Anna with rooting media, we obtained roots in two samples using IAA hormone, this result relatively agreed with (Druart, 1997). The fourth shoots contaminated in this experiment because the inaccuracy of the conditions required for the propagation of apple seedlings, so we did not reach to the stage of acclimatization and we didn't get statistical analysis at this stage.

Considering the fact that many woody species are difficult to root through cuttings (Tereso *et al*., 2008), adventitious root formation is a key step in micropropagation (De Klerk *et al.,* 1997). Sharma *et al.* (2007) reported that consistent high frequency rooting of apple has been more difficult to achieve than shoot multiplication. Moreover, for successful acclimatization of plants, critical traits are the number of roots per shoot and the length of the roots. Like the multiplication rate, rooting ability is also genotype dependent (Sharma *et al.,* 2007; Yepes and Aldwinckle, 1994).

5. Fourth, fifth and sixth experiment

Our results showed that we did not get any shoots, so we did not reach for rooting stage in these experiments.

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5.6.6 The difficulties faced during this research

1. Inaccuracy of the conditions required for the propagation of apple seedlings.
2. Exposure Growth champers to malfunction and overheating several times.
3. Exposure sterilization device to malfunction several times.
4. Unavailability of materials needed for research in the quantities required.
5. Samples exposed to contamination and oxidation significantly.

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Chapter 6

Conclusion and Recommendation

6.1 Conclusion

Based on the results of the present study, the following conclusions are made:

1. *In vitro* cultures can be established at any time of the year, but early sprouting and maximum plant establishment observed with explants collected in summer or spring than those collected in autumn or winter.
2. Use of AC, CA and AA until production of phenolic compounds cease or reduced to the minimum possible, they are important to reduce the risk of death of cultured buds explants of the Anna apple variety.
3. Shoot initiation was optimal on MS medium containing 1.0 mg/l BAP, 0.1 mg/l IBA and 0.5 mg/l GA3 for Anna variety. This media supplemented with 0.1 g/l AC.
4. Shoot multiplication was optimal on MS medium containing 1.0 mg/l BAP, 0.1 mg/l IBA and 0.5 mg/l GA3 for Anna variety, respectively.
5. Rooting was possible on half strength MS medium containing 0.1 mg/l IAA and 1.0 mg/l IBA for Anna variety.
6. The apical bud and axillary shoot buds are enclosed within the leaf sheath. Selecting these young tissues makes it possible to reduce infection since the apical zone displays better aseptic conditions because of the reduced size of the explant and the small area exposed to the external environment.
7. Present investigations have thus shown that the economically valuable Anna apple could be regenerated *in vitro* via organogenesis. The protocol developed will be useful for rapid *in vitro* propagation of the species and also for the subsequent genetic manipulation studies.

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6.2 Recommendations

Future perspectives, based on the present study, should focus on the following areas:

1. Several authors took advantage of *in vitro* systems with a standard and well-controlled culture environment for using them as tools for studying different physiological, biochemical and molecular processes, speeding up breeding works by short-term selection of different traits under *in vitro* conditions.
2. The effect of other phenolic compound adsorbing chemicals like PVP on initiation potential of the explants should be investigated with respect to AC.
3. Rooting response of multiplied shoots at each sub-culturing stage should be investigated.
4. Use of the already developed micropropagation protocol for large scale production of the Anna apple and distributing it to farmers to increase apple fruit production in Gaza Strip should be given due attention.
5. Research on further optimization of acclimatization should be done particularly for Anna variety to increase the survival percentage during acclimatization.
6. Research on further multiplication of any plant at any time of the year should be done particularly for important crops with high nutritional value and medical plants.

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Chapter 7

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Appendix

Table 1. Data of establishment stage in autumn after one week (group A)

Sample name	Length of explant (cm)	No. of new buds	No. of shoots
A1 1	0.3	1	0
A1 2	0.3	0	0
A1 3	0.3	0	0
A1 4	0.4	1	0
A1 5	0.3	0	0
A2 1	0.3	2	0
A2 2	0.5	3	0
A2 3	0.3	0	0
A2 4	0.4	1	0
A2 5	0.3	1	0
A3 1	0.5	4	2
A3 2	0.6	1	3
A3 3	0.3	1	0
A3 4	0.4	2	0
A3 5	0.5	1	2

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Table 2. Data of establishment stage in autumn after two weeks (group A)

Sample name	Length of explant (cm)	No. of new buds	No. of shoots
A1 1	0.3	1	0
A1 2	0.3	0	0
A1 3	0.4	0	0
A1 4	0.4	1	0
A1 5	0.4	0	0
A2 1	0.4	2	0
A2 2	0.7	5	0
A2 3	0.3	1	0
A2 4	0.4	1	0
A2 5	0.4	1	0
A3 1	0.7	7	3
A3 2	0.8	2	5
A3 3	0.3	1	0
A3 4	0.5	2	2
A3 5	0.7	2	3

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Table 3. Data of establishment stage in autumn after three weeks (group A)

Sample name	Length of explant (cm)	No. of new buds	No. of shoots
A1 1	0.4	1	0
A1 2	0.3	0	0
A1 3	0.5	0	0
A1 4	0.6	1	0
A1 5	0.5	0	0
A2 1	0.6	5	0
A2 2	1.2	8	1
A2 3	0.4	1	0
A2 4	0.5	2	1
A2 5	0.6	2	1
A3 1	2.1	3	8
A3 2	1	6	1
A3 3	0.4	1	0
A3 4	0.7	4	2
A3 5	0.9	5	5

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Table 4. Data of establishment stage in autumn after four weeks (group A)

Sample name	Length of explant (cm)	No. of new buds	No. of shoots
A1 1	0.5	1	0
A1 2	0.3	1	0
A1 3	0.6	0	0
A1 4	0.6	1	0
A1 5	0.5	0	0
A2 1	0.9	5	0
A2 2	1.8	3	1
A2 3	0.7	2	0
A2 4	0.7	3	2
A2 5	1	4	1
A3 1	2.7	3	6
A3 2	1.3	9	1
A3 3	1	3	2
A3 4	1	4	3
A3 5	1.1	3	6

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Table 5. Data of establishment stage in autumn after one week (group B)

Sample name	Length of explant (cm)	No. of new buds	No. of shoots
B1 1	0.3	4	0
B1 2	0.4	3	0
B1 3	0.6	1	1
B1 4	0.5	2	0
B1 5	0.5	4	0
B2 1	1	5	2
B2 2	0.7	0	4
B2 3	0.6	6	1
B2 4	0.8	5	3
B2 5	0.5	4	2
B3 1	0.4	1	0
B3 2	0.8	4	1
B3 3	0.4	6	2
B3 4	0.6	4	2
B3 5	0.8	5	1
B4 1	0.3	1	0
B4 2	0.4	4	1
B4 3	0.4	1	0
B4 4	0.5	2	1
B4 5	0.4	1	2

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Table 6. Data of establishment stage in autumn after two weeks (group B)

Sample name	Length of explant (cm)	No. of new buds	No. of shoots
B1 1	0.5	5	0
B1 2	0.5	5	0
B1 3	0.7	2	1
B1 4	0.7	3	0
B1 5	0.6	4	1
B2 1	1.5	5	4
B2 2	1	1	5
B2 3	0.9	6	2
B2 4	1	4	4
B2 5	0.7	5	4
B3 1	0.8	1	1
B3 2	1	3	6
B3 3	0.5	7	3
B3 4	1	3	6
B3 5	0.9	8	3
B4 1	0.3	1	0
B4 2	0.5	6	2
B4 3	0.4	1	1
B4 4	0.6	3	1
B4 5	0.5	2	2

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Table 7. Data of establishment stage in autumn after three weeks (group B)

Sample name	Length of explant (cm)	No. of new buds	No. of shoots
B1 1	0.5	4	1
B1 2	1	2	3
B1 3	1	0	4
B1 4	0.9	2	3
B1 5	0.9	4	3
B2 1	1.7	6	5
B2 2	1.2	1	6
B2 3	1.8	7	3
B2 4	1.3	4	5
B2 5	1	3	7
B3 1	1.2	9	1
B3 2	1.2	4	6
B3 3	0.7	6	4
B3 4	1.1	2	7
B3 5	1	6	6
B4 1	0.3	3	0
B4 2	0.9	3	5
B4 3	0.3	1	2
B4 4	0.7	2	4
B4 5	0.6	2	3

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Table 8. Data of establishment stage in autumn after four weeks (group B)

Sample name	Length of explant (cm)	No. of new buds	No. of shoots
B1 1	0.6	5	1
B1 2	1.4	4	2
B1 3	1	0	4
B1 4	1	3	3
B1 5	1.1	5	4
B2 1	2	8	5
B2 2	1.5	1	7
B2 3	2.2	6	6
B2 4	1.6	5	6
B2 5	1.4	5	8
B3 1	1.4	7	3
B3 2	1.6	4	7
B3 3	1	7	3
B3 4	1.5	3	8
B3 5	1.4	7	4
B4 1	0.6	4	2
B4 2	1.1	5	4
B4 3	0.6	2	5
B4 4	0.9	3	4
B4 5	0.9	4	3

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Table 9. Data of establishment stage in autumn after one week (group C)

Sample name	Length of explant (cm)	No. of new buds	No. of shoots
C1 1	0.3	1	0
C1 2	0.3	0	0
C1 3	0.4	1	0
C1 4	0.4	1	0
C1 5	0.3	0	0
C2 1	0.3	2	0
C2 2	0.4	4	0
C2 3	0.3	1	0
C2 4	0.4	2	0
C2 5	0.3	1	0
C3 1	0.4	6	0
C3 2	0.3	1	0
C3 3	0.3	1	0
C3 4	0.4	2	0
C3 5	0.4	3	0

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Table 10. Data of establishment stage in autumn after two weeks (group C)

Sample name	Length of explant (cm)	No. of new buds	No. of shoots
C1 1	0.4	2	0
C1 2	0.3	1	0
C1 3	0.4	1	0
C1 4	0.4	2	0
C1 5	0.3	1	0
C2 1	0.3	3	0
C2 2	0.5	6	0
C2 3	0.3	1	0
C2 4	0.4	3	0
C2 5	0.3	1	0
C3 1	0.5	8	0
C3 2	0.3	2	0
C3 3	0.4	3	0
C3 4	0.5	3	0
C3 5	0.4	3	0

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Table 11. Data of establishment stage in autumn after three weeks (group C)

Sample name	Length of explant (cm)	No. of new buds	No. of shoots
C1 1	0.4	2	0
C1 2	0.3	2	0
C1 3	0.4	1	0
C1 4	0.4	2	0
C1 5	0.3	1	0
C2 1	0.4	3	0
C2 2	0.5	6	1
C2 3	0.3	1	0
C2 4	0.4	4	0
C2 5	0.4	2	0
C3 1	0.9	8	0
C3 2	0.6	3	0
C3 3	0.4	5	0
C3 4	0.6	3	0
C3 5	0.5	4	0

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Table 12. Data of establishment stage in autumn after four weeks (group C)

Sample name	Length of explant (cm)	No. of new buds	No. of shoots
C1 1	0.5	2	0
C1 2	0.4	2	0
C1 3	0.4	1	0
C1 4	0.4	2	0
C1 5	0.4	1	0
C2 1	0.6	3	0
C2 2	0.6	7	1
C2 3	0.4	1	1
C2 4	0.5	3	1
C2 5	0.6	2	0
C3 1	0.9	8	0
C3 2	0.6	3	0
C3 3	0.4	5	0
C3 4	0.7	3	0
C3 5	0.6	5	0

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Table 13. Data of establishment stage in winter after one week (group B)

Sample name	Length of explant (cm)	No. of new buds	No. of shoots
B1 1	0.6	2	0
B1 2	0.5	2	0
B1 3	0.4	1	0
B1 4	0.7	3	0
B1 5	0.3	1	0
B2 1	1	4	0
B2 2	0.6	3	0
B2 3	0.4	2	0
B2 4	0.9	4	0
B2 5	1.1	2	0
B3 1	0.7	2	0
B3 2	0.9	3	0
B3 3	0.9	3	0
B3 4	0.5	2	0
B3 5	0.7	1	0
B4 1	0.8	3	0
B4 2	0.7	3	0
B4 3	0.7	4	0
B4 4	0.7	3	0
B4 5	0.6	2	0

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Table 14. Data of establishment stage in winter after two weeks (group B)

Sample name	Length of explant (cm)	No. of new buds	No. of shoots
B1 1	1	3	1
B1 2	0.6	3	0
B1 3	1	5	0
B1 4	1.0	5	0
B1 5	0.5	1	0
B2 1	1.3	6	0
B2 2	0.8	5	0
B2 3	0.5	3	0
B2 4	1.1	5	2
B2 5	1.3	3	0
B3 1	0.8	3	0
B3 2	1.1	4	2
B3 3	1.2	4	1
B3 4	0.5	1	0
B3 5	0.8	4	0
B4 1	0.8	5	0
B4 2	0.7	4	0
B4 3	0.7	4	0
B4 4	0.8	6	0
B4 5	0.8	3	0

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Table 15. Data of establishment stage in winter after three weeks (group B)

Sample name	Length of explant (cm)	No. of new buds	No. of shoots
B1 1	1.2	3	2
B1 2	0.6	3	0
B1 3	1	0	5
B1 4	1.3	2	3
B1 5	0.6	0	1
B2 1	1.3	1	5
B2 2	0.7	3	2
B2 3	0.5	2	1
B2 4	1.1	1	5
B2 5	1.4	2	2
B3 1	0.9	3	0
B3 2	1.1	2	6
B3 3	1.2	1	5
B3 4	0.6	1	1
B3 5	1	2	2
B4 1	0.9	1	5
B4 2	0.7	4	0
B4 3	0.7	3	1
B4 4	0.9	4	2
B4 5	0.8	2	1

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Table 16. Data of establishment stage in winter after four weeks (group B)

Sample name	Length of explant (cm)	No. of new buds	No. of shoots
B1 1	1.2	5	2
B1 2	0.6	3	0
B1 3	1.1	3	5
B1 4	1.6	3	5
B1 5	0.7	1	1
B2 1	1.6	3	5
B2 2	0.9	5	2
B2 3	0.7	2	3
B2 4	1.2	1	5
B2 5	1.4	1	3
B3 1	0.8	2	2
B3 2	1.2	1	7
B3 3	1.3	5	6
B3 4	0.7	2	1
B3 5	1.1	2	3
B4 1	0.9	3	5
B4 2	0.7	3	3
B4 3	0.7	1	3
B4 4	0.9	5	3
B4 5	0.9	2	2

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Table 17. Data of multiplication stage after one week

Sample name	Length of explant (cm)	No. of new buds	No. of shoots
H1 1	1.4	8	2
H1 2	1.2	5	3
H1 3	2	5	4
H1 4	1.8	8	4
H1 5	1.1	3	4
H2 1	1	7	3
H2 2	1.2	7	5
H2 3	0.8	5	1
H2 4	1.1	6	5
H2 5	1.1	3	4
H3 1	1	6	1
H3 2	1.2	3	4
H3 3	1.1	2	3
H3 4	0.8	5	4
H3 5	1	3	5

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Table 18. Data of multiplication stage after two weeks

Sample name	Length of explant (cm)	No. of new buds	No. of shoots
H1 1	1.6	11	4
H1 2	1.2	5	7
H1 3	2	5	4
H1 4	2.4	10	6
H1 5	1.1	3	4
H2 1	1.3	11	5
H2 2	1.2	8	5
H2 3	0.8	6	1
H2 4	1.1	7	6
H2 5	1.1	6	5
H3 1	0.9	5	1
H3 2	1.1	3	4
H3 3	1.1	3	3
H3 4	0.8	5	4
H3 5	0.9	3	4

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Table 19. Data of multiplication stage after three weeks

Sample name	Length of explant (cm)	No. of new buds	No. of shoots
H1 1	1.6	10	5
H1 2	1.3	5	7
H1 3	2.1	5	4
H1 4	2.5	5	12
H1 5	1.2	3	4
H2 1	1.6	13	5
H2 2	1.3	8	5
H2 3	1	5	3
H2 4	1.7	8	4
H2 5	1.5	4	8
H3 1	0.9	5	1
H3 2	1	3	4
H3 3	1	3	2
H3 4	0.8	5	4
H3 5	0.8	3	3

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Table 20. Data of multiplication stage after four weeks

Sample name	Length of explant (cm)	No. of new buds	No. of shoots
H1 1	1.7	12	5
H1 2	1.4	5	7
H1 3	2.2	5	5
H1 4	2.8	8	12
H1 5	1.3	4	5
H2 1	2	11	8
H2 2	1.4	8	5
H2 3	1.1	6	3
H2 4	1.8	8	4
H2 5	1.6	6	7
H3 1	0.8	4	2
H3 2	0.9	3	4
H3 3	0.9	3	3
H3 4	0.8	5	4
H3 5	0.8	3	3

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