T.R.
BOLU ABANT İZZET BAYSAL UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
Department of Biology

MICROPROPAGATION OF *CALENDULA ARVENSIS* L. *IN VITRO* AND DETERMINATION OF THE BIOLOGICALLY ACTIVE CONSTITUTES

MASTER OF SCIENCE

MAHABAT TAHER TAWFEEQ

ACADEMIC SUPERVISOR
Prof. Dr. EKREM GÜREL
ACADEMIC CO-SUPERVIZOR
Assist. Prof. Dr. NADHIM SALIM GHANIM

BOLU, AĞUSTOS - 2022

APPROVAL OF THE THESIS

MICROPROPAGATION OF *CALENDULA ARVENSIS* L. *IN VITRO* AND DETERMINATION OF THE BIOLOGICALLY ACTIVE CONSTITUTES submitted by **MAHABAT TAHER TAWFEEQ** and defended before the Examining Committe Members listed below in partial fulfillment of the requirements for the degree of **Master of Science** in **Department of Biology, Institute of Graduate Studies of Bolu Abant Izzet Baysal University in 29.08.2022** by

Examining Committee Members	**Signature**
Supervisor Prof. Dr. Ekrem GÜREL Bolu Abant Izzet Baysal University	.....
Member Prof. Dr. Mehmet Cengiz BALOĞLU Kastamonu University	.....
Member Doç. Dr. Bahtiyar Buhara YÜCESAN Bolu Abant Izzet Baysal University	.....

Prof. Dr. İbrahim KÜRTÜL

Director of Instıtute of Graduate Studies

ETHICAL DECLARATION

In this thesis dissertation that was properly prepared according to the Thesis Writing Rules of Bolu Abant İzzet Baysal University of the Institute of Graduates Studies, I hereby declare that;

- All data, information, and documents presented in the thesis were obtained in accordance with the academic and ethical rules,
- All data, documents, assessments, and results were presented in accordance with the scientific ethical and moral rules,
- All works that were benefitted in the thesis were appropriately cited,
- No alteration was made in the data used,
- Study presented in this thesis is original,

Otherwise, I declare that I accept the loss of all my rights in case any contradiction that may arise against me.

Based on the plagiarism report that was generated on the date of 02/08/2022 by using predetermined filtrations set by Directorate of Institute of Graduate Studies of the Turnitin programme, a plagiarism detection sofware, the similarity index detected was 22%.

_____

MAHABAT TAHER TAWFEEQ

ABSTRACT

MICROPROPAGATION OF *CALENDULA ARVENSIS* L. *IN VITRO* AND DETERMINATION OF THE BIOLOGICALLY ACTIVE CONSTITUTES
MSC THESIS
MAHABAT TAHER TAWFEEQ
BOLU ABANT IZZET BAYSAL UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
DEPARTMENT OF BIOLOGY
(SUPERVISOR: PROF. DR. EKREM GÜREL)
(CO-SUPERVISOR: ASSIST. PROF. DR. NADHIM SALIM GHANIM)
BOLU, AUGUST 2022
(XIII+ 66)

This work involves the plant reproduction of *Calendula arvensis* using tissue culture technology outside the living body by plant parts, top and growing nodes in the field, and from the target plants. These experiments also study the impact of growth regulators BA and KIN with different concentrations on growth, duplication, and the creation of callus resulting from planting part of the leaves in media with different concentrations of 2, 4-D. The effectiveness of alcohol was tested with different sodium hypochlorite (NaClO) concentrations to sterilize plant parts. The results showed that the effectiveness of sterilization using ethanol at 70% concentration for 30 seconds with the use of NaClO at 10% concentration for 20 minutes was the best method of sterilization and growth. The branch upgrowth 2.2 cm branch/plant parts resulting from growing nodes in a field equipped with an 0.5 mg/L BA after 8 weeks was the highest rate. The number of branches 5.5 branch/ plant parts resulting branch end cultivation from the tissues plants on the medium MS equipped with 0.5 mg/L BA after 8 weeks was the highest. Planting of nodes was produced by tissue mothers in the medium of an 0.5 mg /L BA formed the highest number of 2.8 branches/plant part after two months. The number of branches 4.4 branch/plant part - branch end cultivation resulting from the planting of tissues on the medium MS equipped with 2.0 mg/L KIN after 8 weeks was the highest. Planting of nodes is produced by tissue mothers in the medium of a 2.0 mg/L KIN formed the highest number of branches 4.7 branches/plant part after two months. Planting part of the leaf is produced from growing plants in the field on a medium equipped with 1.0 mg/L 2,4-D and creating the highest wet weight of a callus 1.427 g after 4 weeks of planting. The highest wet weight of callus 1.323 g is gained by planting part of leaf consisting of tissues from mothers on a medium that is equipped with 1.5 mg/L 2,4-D, 30 days later planting. Addition of different levels of brassino acid had effect on the growth and multiplication of callus, as it gave the highest dry weight of callus at a concentration of 0.05 mg/L, which amounted to 0.163 g. The prepared medium with a concentration of 2 mg/L of brassino surpassed it in its salicylic content, which amounted to 45.1 ppm. The results showed that there was an increase in the amount of rutin when including the food medium with a concentration of 2 mg/L of brassino stimulant.

KEYWORDS: *Calendula arvensis*, Brassino acid, Callus, Salicylic acid, Rutin

ÖZET

CALENDULA ARVENSİS L.'NİN İN VİTRO MİKROÇOĞALTIMI VE BİYOLOJİK OLARAK AKTİF BİLEŞENLERİN BELİRLENMESİ
YÜKSEK LİSANS TEZİ
MAHABAT TAHER TAWFEEQ
BOLU ABANT İZZET BAYSAL ÜNİVERSİTESİ
LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ
BİYOLOJİ ANABİLİM DALI
(TEZ DANIŞMANI: PROF. DR. EKREM GÜREL)
(İKİNCİ DANIŞMAN: ASSIST. PROF. DR. NADHIM SALIM GHANIM)
BOLU, AĞUSTOS- 2022
(XIII+ 66)

Bu çalışma *Calendula arvensis* bitki bölümleri arazide yetişen bitkilerin tepe noktaları, büyüme nodları doku kültürü teknolojisi kullanılarak hedef bitkiden üretmeyi içermektedir. Ayrıca büyüme düzenleyicileri olan 6-Benzylaminopurine (BA) ve kinetin (KIN)'nin farklı konsantrasyonlarda bitki büyümesine, hücre çoğalmasına etkileri ve farklı 2,4-D konsantrasyonlarında yapraklardan kallus elde edilmesini incelemektedir. Sterizilasyon için alkol ve sodyum hipoklorit (NaClO) ile testler yapılmıştır. Sonuç olarak, 230 dakika boyunca %70'lik etanol ve 20 dakika boyunca %10'luk NaClO kullanımı etkili bir sterilizasyon için uygun bulunmuştur. 8 hafta sonrasında 0,5 mg/L BA ile muamele edilmiş nodlardan büyüyen bitkilerin dal/bitki kısımlarındaki dal büyümesi 2.2 cm olarak en yüksek oranda ölçümlenmiştir. 0.5 mg/L BA ile muamele edilmiş MS ortamındaki doku kültürlerinden elde edilen bitkilerde 8 hafta sonra dal/bitki parçası sayısı 5.5 olmuştur. Hedef bitkilerden 0.5 mg/L içeren besiyerine nod ekimi sonrasında dal/bitki oranı 2.8 olmuştur. 0.2 mg/L KIN eklenmiş MS ortamına ekimi yapılan dokuların dal sayılarının dal/bitki oranı 4.4 olarak en yüksek 8 haftalık bitkilerde saptanmıştır. 2.0 mg/L KIN içeren besiyerinde büyütülen nodlarda 2 ay sonra en yüksek dallanma 4.7 oranı alınmıştır. Arazide yetişen bitkilerin yapraklarından 1.0 mg/L 2,4-D içeren besiyerinde büyütülen kallusların yaş ağırlığı 1.427'dir. En yüksek kallus yaş ağırlığı ise 30 gün sonra 1.5 mg/L 2,4-D içeren besiyerlerine yapraklardan elde edilen dokulardan 1.323 g olarak elde edilmiştir. Farklı seviyelerde brassino asit ilavesi sonrasında 0.05 mg/L olacak şekilde besiyerine eklenen brassino asit kallus büyümesine ve gelişmesinde etkili olmuştur, bu denemede 0.163 g kuru kallus ağırlığı elde edilmiştir. 2 mg/L brassino asit ile hazırlanan ortamda salisilik asit miktarının arttığı 45.1 ppm gözlemlenmiştir. Sonuçlar 2 mg/L brassino uyarıcı konsantrasyonuna sahip besi ortamlarında rutin miktarında artış olduğunu göstermiştir.

ANAHTAR KELİMELER: *Calendula arvensis*, Brassino asit, Salisilik asit, Rutin

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

LIST OF ABBREVIATIONS AND SYMBOLS

2,4-D : 2,4-dichlorophenoxyacetic acid

BA : 6-benzyl amino purine

cm : Centimeter

et al. : and others

EtOH : Ethanol

g : grams

g/L : Gram per liter

GA(s) : Gibberellin(s)

GA_3 : Gibberellic acid

h : Hour(s)

HCl : Hydrochloric acid

K : Potassium

kg : Kilogram

KIN : Kinetin

Kinetin : 6-furfuryl amino purine

L : Liter

M : Molar

Mg : Magnesium

Min : Minute

ml : Millilitre

mM : Micro molar

MS : Murashige and Skoog

NaClO : Sodium hypochlorite (bleach)

NaOH : Sodium hydroxide

oC : Degrees Celsius

pH : Potential of hydrogen

SA : Salicylic acid

Tween 20 : Polyxyethylene sorbitan monolaurate

ACKNOWLEDGEMENTS

I am greatly thankful to almighty ALLAH for giving me his grace and who enabled me to accomplish this humble work. I also thank and gratitude each of the supervisors, Prof. Dr. Ekrem Gurel in Turkey, Dr. Nazim Salem Ghanem in Iraq for their efforts, their accurate observations, their kind treatment, and their beautiful assistance to accomplish this thesis in the best way. I am thankful to my dear parents, my sisters, my brother my beloved husband, who supports and helps me after ALLAH to complete my master's studies, and to all my friends and relatives.

I also thank Tikrit University, Faculty of Agriculture, Department of Agriculture and Gardens Engineering, for their acceptance of the scientific part of my thesis in the laboratory, tissue planting for their university, especially Dr. Harith Burhan Al-Din Abdul Rahman, Head of the Department, Faculty of Agriculture.

I would like to thank Bolu Abant Izzet Baysal University for granting me a master's degree in my scientific career.

Finally, I would like to thank Dr. Shakir Mahdi Saleh, who had a long history of making this thesis successful.

I sincerely thank the professors, the chairman\woman of the discussion committee, and his members who kindly accepted the discussion and evaluated this thesis.

I thank everyone who taught me even a word or advised me or guidance.

ALLAH is the arbiter of success.

1. INTRODUCTION

Medicinal plants have very important role in the today world due to its uses in pharmacy industry. It is preferable to use natural products in the pharmaceutical industry instead of using chemically manufactured products. As the active substances in plant products are much better than chemically made products, because they are characterized by high quality and effectiveness, require less time and effort, without side effects, as well as future damages (Mahesh, 2008). 80% of the population use plant products as a main source of treatment because the developed countries revealed the future side effects of chemically manufactured medicines, and as the World Health Organization (WHO) indicated that 20% of the population of the third world countries are able to buy medicines to treat diseases (Sasson, 1991). In general, natural products have a major role in the development of pharmaceutical industries (Bharathi et al., 2010). Some bacteria have the ability to acquire resistance to chemically synthesized drugs used as therapeutic agents (Towers et al., 2001). Many researchers have confirmed the significant effect of plant extracts on bacteria (Reddy et al., 2001).

Due to the importance of medicinal plants, researchers study them to learn about their uses and benefits, prevent them from losing plant diversity, discover their damages, and know the active substances through the growth of part of the plant (the top, node, stem, leaf) by using tissue culture and precise duplication techniques. These techniques are very important because they are responsible for providing the right conditions for the plant to grow in each season of the year, which gives the researcher the opportunity to study a particular plant in any time. As well as producing healthy, disease-free plants and evaluating the effect of growth regulators on plant growth. During tissue culture, we can observe that the plant has an extrinsic ability to self-division, and the mother plant coincides with the defined growth regulators used that affect secondary metabolites as well as play a major role in the production of primary metabolites (Yadav, 2012).

Calendula arvensis, belonging to the family Asteraceae, is a medicinal herb that contains chemical molecules that have beneficial therapeutic effects that can be used in health care, but in medically prescribed and scientifically determined quantities. This plant has a sweet smell and is highly effective due to its essential oils. It is worth noting that its hexalytic extracts are used as a fungicide, and its

methanolic extracts are resistant to germs and its organic extracts are anti-germs (Abudunia et al., 2014).

1.1 *Calendula arvensis*

1.1.1 Plant Description

It is a species of Calendula and is an annual medicinal herbaceous plant with ornamental features that grow alongside the edges of roads and orchards tall (10-100 cm) (Heyn et al., 1983). It has velvety yellow and orange or yellow vertically rounded flowers that bloom between March and July of each year, which can be used as a spice, food, and tea dye, as well as in the manufacture of cosmetic creams or mirror (Rejšková et al., 2010). It reproduces by seeds, in which the shape of the seeds is relatively large, whimsical, resembling a small crescent, with a high botanical capacity, growing at the beginning of spring, the leaves of which are spear-like, round-tinged with fine hairs with a width of 5 - 20 mm. It has a flat or creeping stem on the ground and has a height of 5-60 cm (Ruiz de Clavijo, 2005). A series of investigations or studies have shown that the external parts of this species contain a high percentage of Siskiterpine glycosides, due to their great Udismani, Luromadendrin, and Copepan (De Tommasi et al., 1990). Numerous chemical studies have proven that this species contains various compounds such as flavonoids, phenolic acid, and linolenic acid, as well as consists of the following components: saponins, sesquiterpene glycoside, triterpenoids, flavonoids, phenols, and saponins (Ahmed et al., 1993, Paolini et al., 2010).

1.1.2 Scientific Classification

The scientific classification of *Calendula arvensis* is given in Table 1.1.

Table 1.1. The scientific classification of *Calendula arvensis.*

Kingdom	Plantae
Clade:	Tracheophytes
Clade:	Angiosperms
Clade:	Eudicots
Clade:	Asterids
Order:	Asterales
Family:	Asteraceae
Genus:	Calendula
Species:	*C. arvensis*

1.1.3 Regions and Plant Distribution

Scientific sources indicate that the origin of *Calendula arvensis* is the southern European regions, which include Turkey, Portugal, Malta, Italy, Spain, and Greece (Rejšková et al., 2010). Also, it is expanded in the Mediterranean basin of the Levant, the Maghreb, and the Caucasus, and so far it is distributed in many temperate regions of the world based on the commercial value. (Muley et al., 2009). This genus includes about 12-20 species (Coste, 1937). The common name for Calendula arvensis is field cutter and is widespread in central and southern Europe, southwest Asia, northern Africa, and the Macrotriaz region (Heyn et al., 1983).

1.1.4 Medical Uses

The parts used in the medicinal treatment of *Calendula arvensis* are the leaves and flowers. Previous studies indicated that the extract of n-hexane and methanol contained in the flowers are highly effective and antibacterial. Their extracts can also be used as a preservative for processed foods, as well as in medicinal and pharmaceutical treatments for infectious diseases. (Aboudonia et al., 2014). In addition, plant extracts can be used as antispasmodics, antiseptics, and diuretics (Tiwari, 2008). The leaf extract has demonstrated antibacterial properties (Jamal et al., 2014), and has been used to treat burns (Passalacqua et al., 2007). Previous studies on anti-inflammatory, antioxidant, and anti-mutagenic activities have also been reported (Arora et al., 2013; Tosun et al., 2012; Ercetin et al., 2012). Previous study also indicated that ethyl acetate extract obtained from *Calendula arvenesis* flowers had cytotoxic activity against breast cancer cells. By its effect on concentration-dependent cell shrinkage, cell detachment, nuclear fragmentation, and chromatin condensation (Abutaha et al., 2019).

1.2 Phytochemical Compounds

The process of metabolism includes anabolic and/or catabolic processes as the chemical changes that occur to the plant within its living cells, structural metabolism processes include the formation of complex organic secondary materials from simple raw materials. In demolitions, complex compounds are broken down and analyzed into simple raw materials (Irchhaiya et al., 2015).

Two Types of Metabolic Compounds are Present:

- The first type of compound is produced by plants: Primary metabolic compounds assist in the processes of plant growth, development and reproduction which are the basic physiological processes of the plant life cycle. Chemical compounds such as carbohydrates, fats, and proteins produced by plant cells in large quantities that directly participate in metabolic processes and plant growth and building the various components of the cell. These compounds are produced by plant cells through the process of respiration and photosynthesis, and compounds such as fatty acids and vegetable oils are used as food and industrial materials in the manufacture of detergents, soaps, dyes, and carbohydrates such as starch, pectin, sucrose and cellulose (Vanisreel et al., 2004).

- As for the second type of plant-produced compounds know as secondary metabolites or complex phytochemical compounds that have an indirect effect in growth, development, and reproduction processes (Shohji et al., 2002).

They are a broad group of low molecular weight organic complexes that have a primary effect on the plant but are not important for plant survival. Natural end products of primary metabolism do not participate in primary metabolic activity, such as phenols, alkaloids, and oils (Kabera et al., 2014). The production of secondary metabolites depends on the developmental stage of the plant, the part of the plant used, and the environmental conditions to which it is exposed, and the process of extraction and purification of secondary compounds is more difficult compared to primary products. The metabolism of these compounds is produced by the leaves of the plant in low concentrations (Dhanarasu, 2012).

1.3 Active Chemical Compounds in the *Calendula arvensis* Plant

1.3.1 Salicyclic Acid

It is one of the important phenolic compounds 2-hydroxybenzoic acid (Dempsey et al., 2011; Lefevere et al., 2020). Phenolic compounds contain an aromatic benzene ring with one or more hydroxide groups produced by plants and a few living microorganisms. As secondary metabolites in nature (Van Hung, 2016; Cheynier, 2012), plants and microorganisms (bacteria and fungi in particular) produce phenols, but in different proportions (Mandal et al., 2010). Phenols in plants play a crucial role in various physiological and biochemical processes (Wallis and Galarneau, 2020), and differences in basal levels up to a hundredfold

between the same types of plants (Raskin, 1992; Seyfferth and Tsuda, 2014). In the late nineties, salicylic compound was accepted as the sixth major plant hormone (Raskin, 1992) and it has different physiological roles (Dempsey and Klessig, 2017; Klessig et al., 2018; Gu et al., 2020), where salicylic acid affects plant growth such as vegetative growth, seed germination, flowering, photosynthesis and respiration (Rivas-San Vicente and Plasencia, 2011; Van Butselaar and Van den Ackerveken, 2020) and it is an important for medical uses (Weissmann, 1991). It was used in the treatment of heart diseases, fever, and vascular diseases (Weissmann, 1991; Dachineni et al., 2017). It is also used in dermatological conditions such as itchy acne and pimples treatment (Arif, 2015) and it is produced as natural product (Grobelak and Hiller, 2017). Salicylic is used as an analgesic (Buchner, 1828) and in 1987 a report on salicylic signals in plants was first published. Also, production was improved when spectral analysis of calla lily flowers (Raskin et al., 1987; Vlot, 2009), and a group of studies showed that high levels of salicylic acid activate genes associated with systemic acquired resistance in some plant species (Métraux et al, 1990; Klessig, 2018). Salicylic acid reduces the stress and toxicity of heavy metals such as copper for its role in abiotic stress (Strobel and Kuc, 1995). It increases the biomass of some plants by decreasing oxidative damage caused by cadmium (Metwally et al., 2003) and acts as an antioxidant (Buchner, 1828). Salicylic plays a major role in activating the immune system of some plants, and it reduces biological stress through genetic response with the partial and biological synthesis of the salicylic compound (Shi et al., 2019).

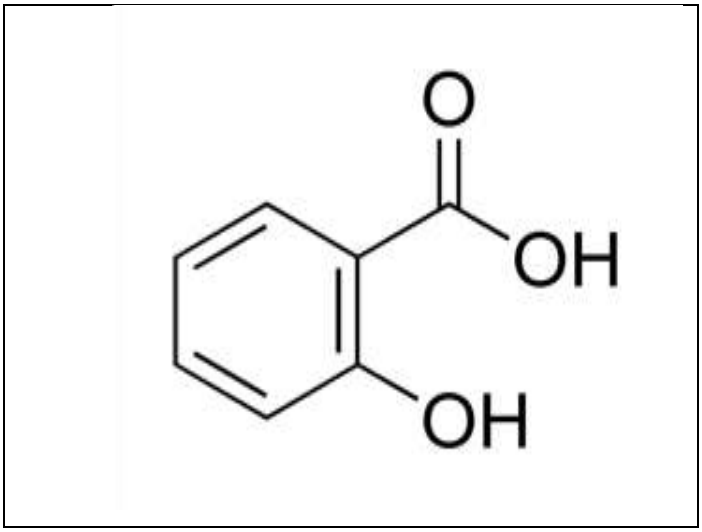

Figure 1.1. The structure of salicylic acid (Lee et al.,1995)

1.3.2 Rutin

Rutin is one of the flavonoid compounds that exhibits a wide range of biological effects (Santos-Buelga and Williamson, 2003) and is one of the most biologically active flavonoid compounds (Mauludin et al., 2009). Flavonoids are naturally found in the form of diphenylpropanoid, which is one of the components of the food system that is available in all places plant foods (Lin et al., 2001) are natural compounds with medicinal properties, rutin is a common flavonoid found in various fruits, seeds, vegetables, nuts, and red wine (Hertog et al., 1993a; 1993b; Formica and Regelson, 1995; Beecher et al., 1999) and rutin acts as antiviral (Davis et al., 2008) and antioxidant (Kumar et al., 2008; Yao et al., 2010; Gupta et al., 2010). Through this property, it can reduce the damage caused by hemi, a highly toxic product that breaks down hemoglobin and excretes hemoglobin. During hemolysis of erythrocytes in hemorrhagic stroke (Yang et al., 2008; Yang et al., 2014; Basu and Kumar, 2014). It is better to use antioxidants than synthetic ones because synthetic antioxidants may cause health problems in the future (Hou, 2003; Prior, 2004). In the laboratory, the antioxidant activity of rutin is similar to that of ascorbic acid (Yang et al., 2008) or higher than that of the alpha to copherd compound (Frankel and Meyer, 2000; Guardia et al., 2001).

It is used in the treatment of heart diseases (Laemmel et al., 1998; Erlund et al., 2000; Nakamura et al., 2001) because rutin acts by blocking arachidonic acid, which reduces capillary permeability and increases its resistance (Alvarez et al., 2003) and has other biological activities. It is anti-cancer and neuroprotective (Kim et al., 2005; Gullón et al., 2017) and reduces cytotoxicity that is associated with chronic disease and cell aging (Bravo, 1998). It prevents human lymphocytes from being damage by protecting cells against mutagens (Duthie et al., 1997). It has other therapeutic effects such as antispasmodics and hypolipidemia (Mauludin et al., 2009), and rutin enhances the solubility of insoluble substances, as well as stabilizing substances, against oxidation, heat, and light, modifying taste by masking flavors, controlling volatility and sublimation, and controlling the production of flavors and drugs (Del Valle, 2004). Several patients use natural medicinal plant products as alternative medicines to supplement medical care for therapeutic purposes (Reis and Mariot, 1999). Alkaloids are extracted from different types of plants and then introduced into clinical treatments due to their low

toxicity and antitumor effects (Huang et al., 2004). Because of their effect on the stability of mast cells, it was used in the treatment of asthma (Geetha et al., 1981).

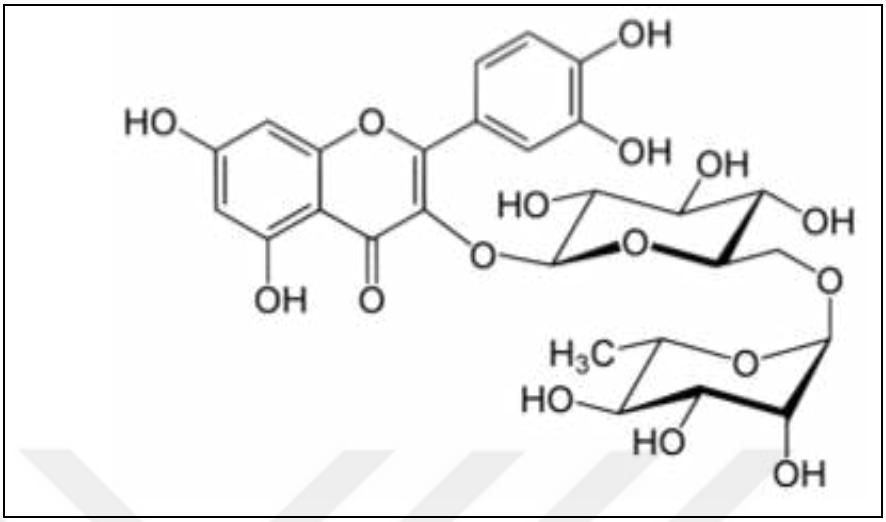

Figure 1.2. The structure of rutin.

1.4 Tissue Culture Techniques or Growing up outside a Living Body

Tissue culture techniques are an important method for the propagation of many species of plants through explants such as leaf, coleoptile, nodes or branches creating new callus through any part of the plant, the growth, and differentiation of calves into branches, and the production of plants through rooting branches. The interest in plant tissue culture has increased in recent decades because it is an important technique for breeding, improvement of plant species, and solution of genetic problems (Ioannou, 1989; Hartmann et al., 1997).

Hormonal control is also an important step in the production of tissue transplants in terms of protoplast integration, increased flour, and production of binary and mono-chromosomal plants. Auxin and cytokinin growth regulators also play an important role in the growth, reproduction, and rooting of branches (Muhammad and Mubashir, 1990). Tissue culture has been very successful in the past 50 years (Gamborg, 2002). Fragments are reserved under strict standard conditions which are provide by climate chamber. Sucrose and plant agar are used for carbon source and solidification of the media, respectively (George et al., 2008; Kumar and Singh, 2009). This technology supports tissue culture on the basis that the ability of living plant cells to divide and produce complete plants identical to the mother plant by cultivating one of the parts of the mother plant if the special

conditions are suitable for organic matter, heat, growth, and humidity, light. In some countries of the world, interest in tissue culture technology has increased because genetic and spontaneous changes can be generated to produce some types of secondary metabolic compounds or increase production through aesthetic changes (Nalawade and Tsay, 2004). Tissue culture is an important method that helps to produce and accumulate chemical compounds such as phenol, alkaloid, soap, glucoside, antibiotic, and resin throughout the year under any environmental conditions and in all geographical locations. As well as establishing tissue culture farms free from fungal and insect injuries, pollution and high purity formative substances. For this reason, this medium is used to extract medicinally effective chemical compounds (Hopkins and Huner, 1995; Lila, 2005). Through tissue culture, sterile, disease-free, and contaminated plants are produced in the laboratory rather than in the field. Embryos may be produced instead of using seeds through protoplast fusion, and hybrid plants may be propagated instead of pollination and feeding (Al-hadidi, 2002; Karuppusamy, 2009). In recent years, researchers have developed several strategies that have a very significant impact on cell production outside the living plant body by selecting the optimal nutrient medium, manipulating the environment, concentration and type of growth regulators as a medium used for some primers using stimuli of specific concentrations and appropriate concentrations utilize of bioreactors (Namdeo and Abraham, 2008).

1.5 Sterilization Stage

It is the first and important step in tissue culture where the plant parts, used tools, and culture media are sterilized after preparation and then the plant parts are grown under suitable and sterile conditions (Salman,1988). Successful, sterile tissue cultures can be obtained after removing the microorganisms and pathogens that cause major problems to the transplanted plant parts by releasing their toxins that harm plant cells, as well as consuming components necessary for plant growth and development causing unsuccessful and damaged tissue cultures (Ramawat, 2004).

In 2005, Grzegorczyk et al. companions sterilized the seeds of *Salvia oficinalis* mermaids by sterilizing 1% sodium hypochlorite (NaClO) for 15 minutes. In 2009, Thirukkumaran and colleagues were able to decontaminate the seeds of *Petunia hybrida* by sterilizing them with 70% ethanol for 30 seconds, then immersing them in 1% NaClO using 20 drops of Tween-20 for 10 minutes, and then

washing them with sterile water. In another previous study, Jaze (2013) confirmed that leaves of *Nerium oleander* sterilized with NaClO, at a concentration of 6.5% for 5 minutes and 10 minutes then washed with sterile distilled water. Al-Hasani (2013) and Al-Fatlawi (2014) obtained sterile seeds of the eyeball plant when sterilized with 1.5% NaClO solution for 15 min. Also, Al-Zuhairi (2016) showed in his study, that obtaining the pollution-free seeds of the cat's eye plant by sterilizing them with 4.5% NaClO solution for 20 minutes and washing them with distilled water 4 times for 5 minutes, and then leaving the plant in distilled water for a period of 20 minutes to ensure that the sterile effect has been removed. Kazemi and Kaviani (2017) indicated that *Petunia hybrida* can be sterilized by washing the seeds under running water with droplets of hand detergent liquid and then immersing those seeds in a 30% H_2O_2 peroxide solution with the addition of 20 drops Tween20 for 10 minutes and then washing them with sterile distilled water three times. Al-Musawi (2017) showed that the best concentration of NaClO solution in sterilizing *Tanacetum parthenium* seeds was 1.2% for 15 minutes with two droplets of Tween-20 dispersing agent, then washing the sterile distillate three times to remove the seed surface from the sterilizer to prevent damage. Habib et al. (2018) confirmed that *Petunia hydrida* was sterilized with running water to remove soil and microorganisms and then washed with Lapolene solution (which is used to clean laboratory instruments) followed by washing with sterile distilled water several times to remove the effect of cleaning materials, then NaClO was added at a concentration of 2% for 5 minutes and then washed with sterile distilled water several times. Mehri et al. (2018) showed through their study of *Petunia hybrida*, 'Opera Super Pink Morn', that sterile and contamination-free seeds can be obtained by washing them under running water with some drops of washing up liquid for 20 minutes and then submerging them at 30% hydrogen peroxide, then adding one or two drops of Tween-20 for 10 minutes followed by washing with sterile distilled water three times 1,3,5 minutes respectively, then immersing in a solution of 20% NaClO by adding a few droplets Tween20 - for 20 minutes. Then wash them with distilled water three times, 1,3,5 minute, respectively. Habas et al. (2019) were able to sterilize *Petunia hybrida* and obtained optimum growth of 90% using 8% NaClO solution for 5 minutes and then washed with sterile distilled water to remove traces of the complex sterilizer. Tawfik and Taha (2019) obtained a sterile *Petunia hydrida* using 50% ethanol alcohol for five minutes and then washed them with sterile

distilled water three times for five minutes each, respectively. Vakili et al. (2019) reported that it is possible to obtain contamination-free seeds upon immersion - *Petunia hybrida* seeds in 70% ethanol, for half a minute, 1% NaClO for 10 min, and washed with sterile distilled water three times.

1.6 Gibberellins

Gibberellins (GAs) were discovered in Japan in 1930 when scientists and farmers studied rice disease. The skeleton of Gibberellins is made up of 20 or 19 carbon particles (Stirk et al., 2014). The formation of GA is produced by plants and fungi from Geranyl Diphosphate by reproduction. 140 species of Gibberellins have been isolated (Kawaide, 2006), but only three are commercially available and have biological properties; GA_4, GA_7, and GA_3.

GA_3 is a tetracyclic C1-C2 Hydroxy Lactone double acid with an OH at C13 and containing a double C10Y-lactone group. It is a white crystalline powder soluble in alcohol, ethyl, acetone and butyl ores. Its melting point is 235-233 °C, it decomposes at high temperatures, and it has an alkaline pH. It is stable in dry conditions. It is involved in the growth and development processes of many plants (Lovegrove et al., 1998).

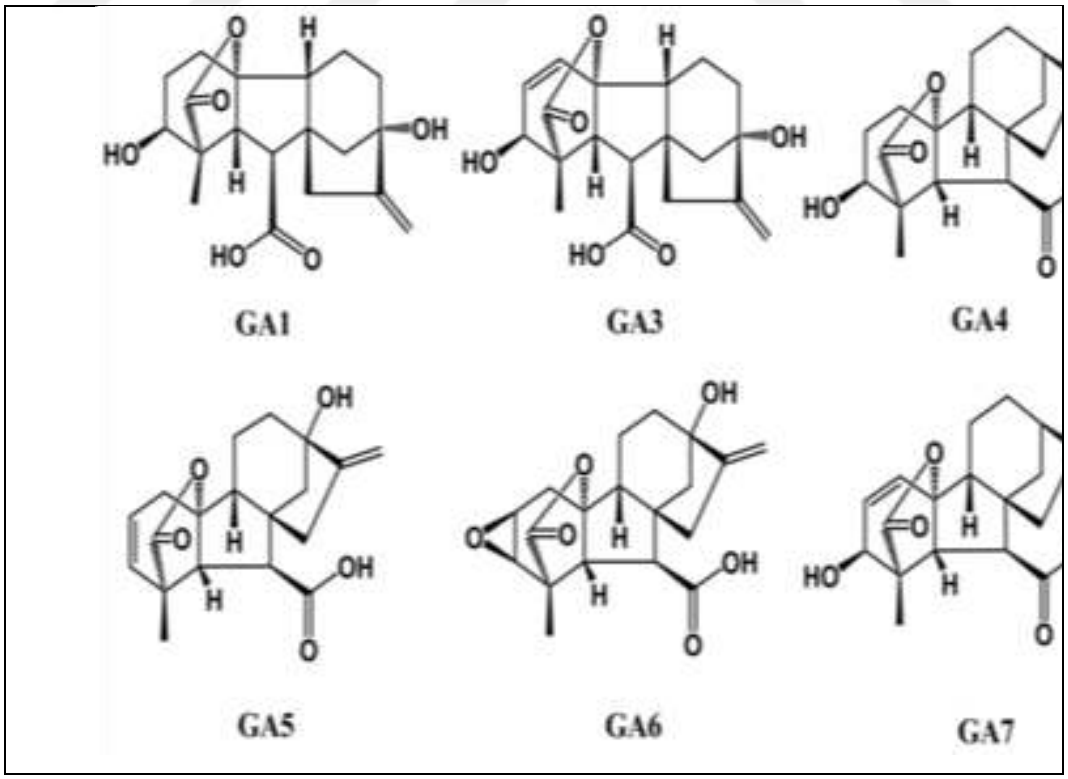

Figure 1.3. The structures of gibberellins (Han et al., 2018) .

1.7 Effect of Cytokinins on Evolution and Multiplication of Branches

Cytokinins are plant hormones that terminate the plant's static phase, and they are nitrogenous bases with high partial weights that have a role in determining the persistence or cessation of plant growth (Dello loio, 2007). They are produced by a plant in a very small amount in most of its parts, such as the roots, leaves, buds, and fruits. Its effect appears in the same parts of the plant as it occurred or in any part of the plant. Cytokinins vary in response to duplication and adenine is one of the most active and most frequently used cytokines for plant parts transcription (Attia and Judua, 1999). On the other hand, they activate cell division, production of new cells, and development of armpit buds by ending the top sovereignty of branches, breaking seed dormancy, and stimulating the formation of broad branches from the chalice tissues (Al-Rifai and Al-Shobaki, 2002, Ramawat et al., 2004). An important stage that plays a major role in the process of plant reproduction is the stage of development, which is the highest rate of growth and duplication with the use of an appropriate nutrient medium.

Several scientific studies have confirmed that the source of cytokinins is either a natural plant such as zeatin, which consists of N; D. ribos substrate for phosphoric acid, or chemically synthesized in laboratory Thidiazuronuriabenzel Adenine (TDZ) (George et al., 2008). The success of duplication depends on the type of cytokinin, and the plant part used. Qassab Bashi and Yahya (2017) found that planting the limps of branches of *Calendula oficinalis*, a lemon kingdom class in the middle of Murashige and Skoog medium, 1962 (MS) equipped with different groups of 6-Benzylaminopurine (BA) resulted in the highest branch rate 7.8 branches/plant parts, 2.7 cm long and leaf rate for longest branch 7.7 leaf/plant. Branch when planting on 1.5 mg/L BA medium 8 weeks after planting. When transplanting nodes on MS medium prepared with 1.0 mg/L BA resulted in the highest branch rate of 3.6 branches/plant, length of 2.8 cm, and leaf rate of longest branch 7.9 leaves/branch after 8 weeks of cultivation.

Karim et al. (2002) reported a maximum rate of 4.2 substrate/plant part when growing 5.8 cm long Daoudi (*Chrysanthemum* m*orifolium*) branch tips, 5.3 cm/branch contract rate when growing in mid-MS equipped with 1.0 mg/L BAP and the highest reaction rate of 91%. While the maximum number of branches/plant part was 3.5, with an average of 4.2 cm and a length of 4.1 nodes implanted on MS

medium with 0.5 mg/L kinetin (KIN) and the highest 60% response after 6-8 weeks of culture.

Therefore, during the growth of the same plant nodes in the middle of MS, equipped with 1.0 mg/L BAP, it obtained the maximum number of branches, 5.3 branches/plant part, 7.0 cm long, nodes/branch 7.5 cm, and generated the highest 95% response rate. It had the highest number of branches 2.8 plant branches/parts, the highest number of branches 2.8 plant branches/parts, an average of 3.5 cm, and a contracting rate of 4.0 knots/branch when grown on prepared MS medium equipped with 1.0 mg/L KIN and gave the highest response rate of 66% after 6-8 weeks of cultivation. According to Abu – Qaoud et al. (2010), the peripheries of *Petunia hybrida* branches obtained from culture mounted on MS medium with 0.8 mg/L BA yielded the maximum number of branches (7.8 branches/plant). Four weeks after planting, the maximum number of leaves was 25.0 leaves/plant and parts was 3.8 cm long. Qassab Bashi and Al-Mamari (2010) investigated the replicating of two species of Dianthus caryophyllus and Jeanne Doinis Blano. Where Yellow Flower Marie Chaboud Jaune reacted with Sibean agriculture guaranteed plant and got the best doubling of the limbs of white species in MS center equipped with 8.0 mg/L of KIN, with 16.90 branches/plant parts. After 8 weeks of culture, the yellow half gave 14.40 branches/plant fractions with a KIN concentration of 6.0 mg/L. In 2011, researchers demonstrated that branch limb doubling of Dianthus caryophyllus reacted with 100% tissue culture for both classes and at an average of 23.10 branches/plant as part of yellow branch cultivation. On MS medium equipped with 3.0 mg/L BA, white branch parts yielded 21.0 plant branches/part of those grown on medium supplied with 1.0 mg/L BA after 8 weeks of cultivation. As Qassab Bashi and Al-Mamari (2011) reported from their motifs on the carnivorous Dianthus caryophyllus that the white-flowered Jeanne Doinis Blano class, and the yellow-flowered Mary Chapaud Jaune class, increasing the nodes of the white species at 4.0 mg/L KIN gave the mean the largest number of branches 12.40 vegetative branch/parts, while the highest number of branches was 12.70 branches/parts of zero-type nodes that grew on the medium of 8.0 mg/L KIN after 8 weeks of cultivation. According to the researchers (2011), the nodes of Dianthus caryophyllus broccoli responded to tissue culture 100% for both classes, with the largest branch rate being 20.30 plant branches/parts of the nodes of the white species cultured on medium MS equipped with 1.5 mg/L BA. While the

largest number of branches/parts of plants grown with yellow contract was 18.30, on the same medium after 8 weeks of planting. During 17 days of culture, Nalini and Nagar (2012) had the best branch development response with 67.82 percent endocytosis of chrysanthemum myrophila, on medium MS equipped with 3.0 mg/L KIN overlapping with 2.0 mg/L Indole-3-acetic acid (IAA).

Abbasi and Qassab Bashi (2013) were able to develop the limbs of *Digitalis purpur*ea branches obtained from tissue culture on MSALs medium with 0.75 mg/L BA, yielding the largest number of branches 8.6 branches/cm long with a length of 3.04 cm. The largest proportion of branches was 6.1 plant branches/parts, with an average length of 2.33 cm when grown on a medium equipped with 4.0 mg/L KIN after 8 weeks of cultivation. They also discovered that the cultivation of digitalis purpurea nodes from a textured culture in the middle of MS with 0.75 mg/L BA resulted in the largest number of branches 18.8 branches with a length rate of 2.64 cm and a leaf rate of 121.3 cm. The greatest number of branches was 7.1 plant branches when grown in mid MS at 1.0 and 4.0 mg/L KIN 8 weeks after planting. According to Al-Mamari (2014), implantation branches limbs of *Catharanthus roseus* on prepared MS medium: 0.75 mg/L BA resulted in the highest branch ratio of 2.70 branches/part, the average height of 1.70 cm, and the average number of leaves 8.40 leaves/branch. After 8 weeks of culturing, the number of branches growing in the center of MS equipped with 4.0 mg/L KIN increased to 4.80 branches/parts and an average length of 2.65 cm.

Al-Mamari (2014) discovered that growing Catharanthus Roseus at medium MS with 1.0 mg/L BA resulted in the largest number of branches (2.90 branches/plant part), an average length of 2.75 cm, and an average number of 9.40 leaves /branch. It exhibited the highest rate of 4.20 branches/plant parts and the best doubling of its culture on 4.0 mg/L KIN medium. It is 2.70 cm long and averages 8.60 leaves/branch after 8 weeks of planting.

Al-Badrany (2014) investigated the possibility of doubling the ends of the branches of the Daoudi plant. Chrysanthemum morifolium, Yellow Princess Anne, as a tissue plant, when grown on MS medium containing 1.0 mg/L BA, showed the highest branch rate (15.11 branches/plant part) and the longest length (5.66 cm). The average number of leaves was 73.0 leaves/plant part, while the highest rate was 2.90 branches/plant part and 5.90 cm in cultivation on a medium of 3.0 mg/L KIN after 8 weeks of cultivation.

Growing of *Chrysanthemum morifolium* class, Yellow Princess Anne from tissue culture on MS medium equipped with 2.0 mg/L BA produced a maximum multiplication rate of 5.53 branches/plant part and 6.16 cm in length, according to Al-Badrany (2014). The total number of leaves was 32.26, with a maximum multiplication rate was 4.40 branches/plant part, the length was 5.45 cm and the average number of leaves was 33.20 leaves/plant part when grown on MS medium was equipped with 4.0 mg/L KIN after 8 weeks of cultivation. According to Al Hayani (2014), the edges of *Dahlia variabilis* L. branches after 4 weeks of culture, average MS with 3.0 mg/L BA produced a maximum number of 4.0 branches/parts.

Al-Hayani (2014) indicated that the highest number of branches was 4.26 branches/plant parts of *Dahlia variabilis* L. In the medium, MS was equipped with 1.0 mg/L BA, while the highest number of branches was 5.57 branches/ton from cultivation and MS was equipped the middle was 7.0 mg/L KIN 4 weeks after cultivation.

According to Aminnejad et al. (2015), branch end culture of *Hyoscyamus reticulatus* L, obtained from fields from textured cultivation on MS medium prepared with 5.0 mg/L BA with 0.1 mg/L IAA after 9 weeks of cultivation, plant branches produced 10.14 branches plant part.

Aminnejad et al. (2015) showed that tissue culture on the medium MS with 1.0 mg/L BA and 0.1 mg/L IAA resulted in the cultivation of the *Hyoscyamus reticulatus* L. plant contract collected from paddies. After 9 weeks of cultivation, the plant branches produced 8.56 branches/plant parts.

Ibrahim and Daraj (2015) discovered that the edges of the branches of *Dahlia variabilis*, obtained from textural paddies and grown on MS medium with 2.0 mg/L BA, overlapped with 2.0 mg/L NAA. After 8 weeks of culture, NAA yielded the largest branch rate of 5.0 branches/plant part with an average length of 3.33 cm and an average leaf count of 5.33 leaves/branch.

According to Abed Elmaksood et al. (2016), cultivation of *Hyoscyamus moticus* L. plant contract, taken from paddies in MS medium equipped with 1.0 mg/L of KIN, resulted in the highest tissue culture response rate of 100% and the highest rate of 8, plant branch/ portion, 1.37 cm long 4 weeks after planting. The number of branches formed in each plant fraction increased gradually by re-seeding on the same medium, with 100% of branches formed, 12467 branches/plant parts of 2.298 cm in length 4 weeks after replanting.

Growth of the growing apex with the phylogenetic part of *Withania somnifera* L. which is used from paddies from cultivation grown on MS medium prepared with 1.0 mg/L BA, gave the highest branching rate 4.22 branches/plant parts after 30 days of cultivation according to (Hamood and Majeed, 2017).

According to Marcinek et al. (2019), the peripheries of Dahlia (pirat) branches grown on MS medium equipped with 0.25 mg/L BA had the highest number of branches 3.8 plant branches/part, while MS medium contained the largest number of branches 2.4 branches/plant part. After 6 weeks of cultivation, it was equipped with 2.0 mg/L KIN.

According to Al-Mukhtar et al. (2019), the development of bazon-eye branches *Catharanthus roses* limbs on medium MS with 3.0 mg/L BA overlapping with 0.5 mg/L IAA resulted in the highest rate of 21.66 branches/plant parts with a length of 7.0 cm and most branches (15.66 branches/plant parts). At a length of 5.66 cm when cultured on 3.0 mg/L KIN medium overlapping with 0.5 mg/L IAA one month after cultivation.

After 6 weeks of cultivation, Marcinek et al. (2019) found that growing dahlia (pirat) on MS medium equipped with 0.25 and 2.0 mg/L BA resulted in the highest branch rate of 4.3 plant branches/part, while growth on MS medium equipped with 1.0 and 4.0 mg/L Kin resulted in the highest branch rate of 1.8 branches/plant fraction.

Bashi (2020) discovered that after 8 weeks of cultivation, planting branches generated by tissue culture on MS medium equipped with 2.0 mg/L BA had the greatest branching rate of 9.3 branches/plant part. While tissue culture on middle MS equipped with 1.0 mg/L BA yielded the most branches 10.2 branches/plant part of nodes) 8 weeks after cultivation.

Almemary et al. (2020) reported that growing *Dalia hydridae* in MS medium at a dose of 2.0 mg/L BA resulted in the highest branch rate of 2.50 branches/plant part, with a length rate of 1.15 cm and an average number of leaves of 9.12 leaves/plant part, and the highest percentage of branches was 2.25 branches/plant parts, with a length rate of 1.48 cm and an average leaf count of 6.50 leaves/plant parts, of their cultivation on a 4.0 mg/L KIN medium after 8 weeks of cultivation.

On the 0.5 mg/L BA media, Almemary et al. (2020) produced the best doubles for the *Dahlia hydrida* nodes, which stopped at 2.87 branches/plant part,

1091 cm in length, and 11.75 leaves/plant part. On a 4.0 mg/L KIN medium, the maximum number of branches was 4.0 plant branches/parts at a length rate of 1.73 cm and a leaf count rate of 8.87 leaves/plant parts after 8 weeks of cultivation.

According to Al-Dabagh and Salih (2020), the most important component of branch development was 80% of the branch-end culture of the plant. *Salvia hispanica* L. harvested from textured cultured paddies on MS medium with 1.0 mg/L BAP. Most of the branches produced 3.20 branches/plant parts with an average length of 3.26 cm. While the maximum percentage of branches was 40% of those grown in MS medium equipped with 1.0 mg/L KIN and the number of branches/plant parts was 1.0 with an average length of 1.86 cm after 8 weeks of culture.

Rokosa and Kulpa (2020) confirmed that *Stevia rebaudiana* plant nodes grown from paddies from tissue culture on MS medium supplemented with 1.0 mg/L BAP were successful. This resulted in the highest branch rate of 3.38 plant branches/parts when grown on 5.0 mg/L KIN medium, and the highest branch rate of 2500 plant branches/parts when grown on 5.0 mg/L KIN medium.

1.8 Growth Regulators in Callus Formation

The callus tissue is a mass of unspecialized, irregularly shaped Brenchemical cells that are strongly opposed to each other. They are fragile and vary in callus activity and growth depending on the type and concentration of the growth regulator, that is, the type of plant part used.

The callus was developed using growth regulators, which include oxygen and cytokinins added to the nutrient environment for growing plants in tissue culture, as well as estimates of the use of active substances in the plant (Al-Kinani, 1987; Hartmann et al., 2002).

Al-Badrany (2014) cultured leaf parts of *Chrysanthemum morifolium* class Anne yellow princess on MS nutrient medium supplemented with 1.5 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) with 0.1 mg/L Indole-3-butyric acid (IBA) 56 days after cultivation. Al-Mamari (2014) received the highest wet calcification rate of 1.749 g by growing a leaf part of *Catharanthus roseus* on MS nutrient medium at 0.75 mg/L 2,4-D after 24 days of cultivation. Khater and Elashtokhy (2015) leaf culture of *Hyoscyamus aureus* L. had the highest callus production rate of 88.6% when leaf was grown on MS medium using 1.0 mg/L 2,4-D.

Elshorbagy et al. (2018) were able to grow a callus-sized *Atropa belladonna* leaf when the leaf was grown in 1.0 and 2.0 mg/L 2,4-D medium 21 days after cultivation.

Whereas, Al-Akedi and Qassab Bashi (2018) were able to obtain from a growing portion of the leaf produced by St. atropappilladonal mothers on a medium supplemented with 1.0 mg/L 2,4-D , a proportion of the introduction of 100% calves and the highest average wet weight of 0.97 g after 28 days of cultivation. They showed the differentiation potential of the developed calyx when replanted on MS medium with 1.0 mg/L 2,4-D. The highest percentage of branches was 1.0 branches/plant part and 10% after 56 days of cultivation.

1.9 The Role of Plant Tissue Culture Technology in the Production of Secondary Metabolites

In the seventies of the last century 1975, it was thought to produce plant secondary metabolites through tissue cultures, and the percentage of achievement was very small in this field, and rapid work began for this technology on different types of plants, including *Panax ginseng* and *Rauwolfia Serpntina* (Al-Rifai and Al-Shobaki, 2002).

The tissue culture technique has been used to increase the production of the necessary secondary metabolic compounds produced from the different explant types. These explants grow on sterile nutrient media supplemented with plant growth regulators and to obtain them in the required quantities in an economical way that cannot be obtained by the usual methods of cultivation. For suspended cell cultures or callus cells, from which compounds are extracted, this technique has provided opportunities to produce several secondary metabolites at any time of the year without being restricted to a particular season. In the possibility of increasing the accumulation and production of secondary metabolic compounds, and a lot of effort has been made to develop advanced protocols for enhancing plant cell productivity, such as ways to change the nutritional environment and the environment, using different types and concentrations of plant growth regulators and appropriate concentrations of stimuli, and many researchers have confirmed that these strategies have an effect in the productivity of plant cells under specific environmental conditions outside the *in vivo*, for example, stimuli that accelerate the production of active compounds, the development of genetic mutations, selection of tissue cells from high- productivity strains, the use of primers as support

for the nutritional medium, one of the basic substances for the synthesis of secondary metabolites , as well as the use of bioreactors and the selection of strains with high productivity or the development of low-productivity species, also the use of different types of lighting (Namdeo, 2007; Abraham, 2008).

1.10 Factors Affecting the Production of Secondary Metabolites

1.10.1 The Effect of Chemical Stimuli on the Production of Secondary Compounds

Through the study of several researchers on the production of secondary metabolites, it was found that it is possible to stimulate the plant to increase the production and accumulation of secondary metabolites by controlling the factors that affect its growth such as physical factors that include (temperature, light, type, composition of the nutrient medium user, and aeration) and nutrients (nitrate or ammonium, nitrogen source, carbon source, phosphate, mineral elements, initiator molecules). They alter plant productivity of secondary metabolites when transplanted cells are treated with external stress (tensile), which varies according to the developmental and physiological stage of the plant.

Several scientific studies and recent developments have shown that organic and inorganic molecules can stimulate plant by-products with regard to secondary metabolism (Zhao et al., 2001a; Zhao et al., 2001b) two steps are taken to achieve an increase the productivity of secondary metabolic compounds, the first by increasing the biomass, and the second by controlling nutrients as the mediator of production. When growth stopped in the production medium, the production resulted from raising the level of sucrose (10.4%) and reducing nitrogen and phosphate (Ramawat, 2004). The researchers also found that the substance is affected when added in low concentrations to the living cell system, which decreases or increases the biological structure of a specific compound known as a catalyst. Depending on their nature, catalysts can be divided into biotic and abiotic stimuli, and depending on their source, stimuli are divided into endogenous and exogenous (Park et al., 2008).

Among the most important catalysts that affect or stimulate the production of secondary metabolic compounds:

1.10.1.1 Brassino

They are plant hormones that have important roles in physiological processes such as plant development and growth, cell division and elongation,

protein and enzyme activation, photosynthesis, and DNA synthesis (Sasse, 2003). Remarkable changes are caused by low level of concentrations on growth and differentiation (Mitchell et al., 1970) showed that brassino not only stimulates stem elongation and cell division, but also increases the total biomass and yield (Nemhauser et al., 2004) and also participates in the development, reproduction and homeostasis of plants (Lösel and Wehlıngö, 2003) increases fertility and seed germination (Taiz and Zeiger, 2003) xylem formation (Zurek et al., 1994), that is, it regulates a number of genes involved in the development of xylem (Gomes, 2011) and plant resistance and prevention of various stresses, i.e. withstand the stress caused by Floods, droughts, salinity, high temperature, oxidative stress, heavy metals (Hayat et al., 2010; Bajguz and Piotrowska- Niczyporuk, 2014; Asha and Lingakumar, 2015) and the ability to confer plant resistance to stress Temperature (Fariduddin et al., 2011, Gomes, 2011) and its lack of effect on healthy cells in addition to preventing the growth and development of cancer cells (Malikova et al., 2008) protects humans from health risks (Thakur and Sohal, 2013) and reduces the effects of pesticides and fungi on plants, and thus reduce the toxic effects of pesticides on plants (Sasse, 2003; Xia et al., 2006) and brassino acid is considered as one of the catalysts for the production of secondary metabolites in plants (Hayat et al. 2010; Johnson and Lingakumar, 2011). Brassino had a significant effect on model plants of economic importance (Lieselotte et al., 2014). It is used plant and agriculture studies. It increases productivity and improves crop quality and stress tolerance of crops (Vriet et al., 2012).

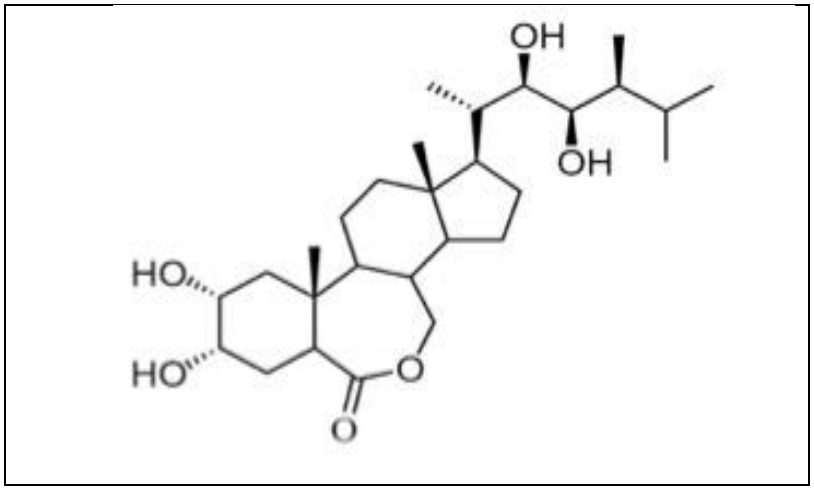

Figure 1.4. The structure of brassino. (AL-Khafaji, 2014)

2. AIM AND SCOPE OF THE STUDY

1- Determination the most suitable explant type for plant formation from the wild *Calendula arvensis*.

2- Determination the most effective growth regulator in the growth of branches and their duplication.

3- Determination of the growth regulator that stimulates callus formation from true leaves explant.

3. MATERIALS AND METHODS

The experiments were conducted on *Calendula arvensis* in Transplantation Tissue and Plant Cells lab attached to the Department of Horticulture and Garden Engineering, College of Agriculture, Tikrit University in the State of Iraq for a period from November 2020 to February 2022.

3.1 Sterilization and Use of Tools

The following laboratory instruments: beakers, colander, Petri dishes, pipettes, scalpels, and distilled water were placed in the autoclave for 20 minutes at 121 °C and pressure of 15 kg/cm^2, and ethyl alcohol at a concentration of 70% was used to sterilize the surgical blades and glues existing inside the hood before exposure to the heat of the flame, and using the Laminar-air Flow Cabinet also hands and glass ends were sanitized before getting into the wood.

3.2 Nutritional Medium Preparation and Sterilization

For all stages of cultivation, which included seed planting, Calendula arvensis growth, and chalice development, one quality of ready-made MS was used for experiments. The source of MS and the organic salts and vitamins needed for plant growth are given in Table 2 and the nutrient medium is prepared with the manufacturer's guidance. To prepare one for medium nutritional, irrigation 400 mL of distilled water was placed in the flask over a hot purple stirrer, to which 4.9 g/L MS was added, and an amount of sucrose was added to 30 g/L until the sugar was completely dissolved. In another flask, 400 ml of distilled water was added, and then 7 g Agar was added. The heater was placed in a continuous movement until it melted, then the two medium solutions were mixed with the acre solution and the volume was completed to 1000 ml with a slender medium, then it was placed on the rotary heater again until boiling and homogeneity of the solution. A growth regulator was added according to the type of study, then the pH of the hydrogen ace was measured and adjusted to 5.7 ± 0.1 by measuring the pH scale by either adding droplets of NaOH if the pH was less than 5.7 or adding droplets of HCl if the pH was higher than 5.8. The medium was distributed into glass bottles and pieces of aluminum foil were used to cover the flaps of the bottles, then the implants were stacked by autoclave at 15 kg. pressure/cm^2 and temperature 121°C for 20 minutes, and after the middle sterilization stage was completed, the floors of the apparatus were taken out and the medium was then left to solidify and was ready to grow after

the temperature of the chamber has been cooled. The nutrient media was then transferred to a Laminar-air Flow hood and all tissue culture experiments were done under sterile conditions.

Table 3.1. Components of the MS nutrient medium.

Ingredients	Chemical formula	Concentration (mg/L)
Macronutrients		
Ammonium nitrate	NH_4NO_3	1650
Potassium nitrate	KNO_3	1900
Calcium chloride hydrate	$CaCl_2$. $2H_2O$	440
Magnesium sulfate hydrate	$MgSO_4.7H_2O$	370
Potassiu dihydrogen phosphate	KH_2PO_4	170
Micronutrients		
Boric acid	H_3BO_3	6.2
Hydrated manganese sulfate	$MnSO_4.4H_2O$	22.3
Hydrated zinc sulfate	$ZnSO_4.7H_2O$	8.6
Hydrated sodium molybdate	$Na_2MoO_4.2H_2O$	0.25
Potassium iodide	KI	0.83
Hydrated copper sulfate	$CuSO_4.5H_2O$	0.025
Hydrated cobalt chloride	$CoCl_2.6\ H_2O$	0.025
Hydrated ferrous sulfate	$FeSO_4.7H_2O$	27.8
Ethylene Diamine Tetrasodium Salt	Na_2 EDTA	37.25
Vitamins and amino acids		
Thymine hydrochloric acid	Thiamine – HCl	0.1
Nicotinic acid	Nicotinic acid	0.5
Pyridoxine hydrochloric acid	Pyridoxine – HCl	0.5
Claicin	Glycine	2
Mayo Inositol	Myo – Inositol	100
Sucrose	Sucrose	30000
Agar	Agar – Agar	6000
pH	PH	5.7-5.8

Distilled water	Distilled water is sufficient to supplement the volume of the nutritional medium to one liter 0.1

3.3 Source of Plant Parts

Two sources of plant parts were used, the first was maternal tissue formed by growing seeds in the laboratory on nutrient media that did not contain a growth regulator, and parts of the plant were used for study. These parts were free from contamination and pathogens and for this reason there was no need for sterilization (Fig. 3.1A). As for the second source, it was the plant parts from the field, which was obtained from the garden of the College of Agriculture, but this source was sterilized before using it (Fig. 3.2B).

Figure 3.1. A) The plant that grows in the lab, B) The plant from the field.

3.4 Plant Parts Used in Tissue Culture Experiments

Plant parts were synthesized by the tissue mothers.

Node: 1 cm long node was used after cutting the leaves to double.

Buds: 1 cm long bud was used after cutting the leaves attached to the buds for duplication.

Leaf: A part of the leaf with a length of 1 cm and a width of 0.5 cm was used after cutting its edges to increase the area of callus formation.

3.5 Plant Parts Formed from Plants Growing in the Field

Node: 1 cm long node was used after cutting the related leaves to double.

Apex: 1 cm long bud was used after cutting the leaves attached to the bud for multiplication.

Leaf: A part of the leaf with a length of 1 cm and a width of 0.5 cm was used after cutting its edges to increase the area of callus formation.

Figure 3.2. The cutting of the plant parts.

3.6 Preparation, Sterilization, and Germination of Seeds

The whole procedure for this part is given step by step,

- The seeds were washed with running water for 30 minutes to remove suspended dust and impurities.
- Then seeds were then soaked with gibberellin at a concentration of 50% for half an hour.
- 70% ethanol was used for 30 seconds and then the seeds were washed with sterile distilled water once for five minutes.

- The seeds were washed with a 10% solution of NaClO for 20 minutes.
- The seeds were washed with sterile water three times for six minutes each time to remove the damage of the sterile material.
- The sterilized seeds were grown in MS nutrient medium free from adding any growth regulator at a rate of 100 refiners each time and several seeds were sown for each refiner.
- The seed was incubated in the growth room at a 25 °C and 8 hours in the dark, followed by 16 hours in the light and the intensity of illumination at 1000 lux.
- The plants were monitored continuously, with notes and results recorded.

3.7 Preparation and Sterilization of The Surface of Plant Parts

The node and apex plant parts were taken from newly grown plants in the field that were free of disease and insect infestation. These parts were then washed under running water for 30 minutes to remove microorganisms and dust, then transferred to the inside of the hood, and then the plant parts were placed in a glass tube with a cap. Then 70% ethanol was added to it for 30 seconds, and then it was emptied of ethanol with the addition of sterile distilled water with continuous stirring for 5 minutes and for one time to remove the effect of alcohol on the plant parts. 10% NaClO was added under constant stirring for 20 minutes (for minor use containing 6% NaClO) as a source of sodium hypochlorite. The plant parts were washed with distilled water 3 times for 5 min each time to remove the effect and damage of sodium hypochlorite and. After the sterilization process, the previously used petri dishes were sterilized and the immobilized plant parts were placed on them. The forceps and the surgical blade were used to cut the plant parts about 1 cm long, cut off the ends of the limbs in contact with the chemicals (sterilization solution), and then the plant parts were ready for cultivation.

3.8 Surface Sterilization of the Leaf and Preparing it for Cultivation

The fully- bloomed, fresh-grown leaves were taken from plants that were growing in the field and free from disease and these parts were placed under running water for 30 minutes to remove suspended matter, dust, and contaminants, then they were transferred to the inside of the hood, and then they were placed in a glass tube with a cover. Then ethanol was added at a concentration of 70% for 30 seconds,

then the parts were washed with sterile distilled water to eliminate the effect of ethanol for one minute and once, then sodium hypochlorite solution NaClO was added at a rate of 10% with continuous stirring for 20 minutes. The plant parts were drained of NaOCI solution and then washed with sterile distilled water 3 times for 5 min each time to eliminate the effect of sodium hypochlorite and to prevent damage to the plant parts. All this was carried out in a sterile medium and under aseptic conditions.

3.9 Extraction of Salicylic Acid from the *Calendula arvensis* Plant

All samples (callus) were placed at room temperature after drying. The dried samples were ground and weighed 0.3 g of sample was carefully ground into a 50 ml tube and cooled 20 ml of methanol was added to it 2 ml of phosphoric acid after the procedure. The speed of centrifugation reached 5000 rpm for 10 minutes, then he organic layer was taken and 10 ml of ethyl acetate was added to it, then 0.6 g of primary secondary amine (PSA) was added and placed on the vortex for 2 minutes and then centrifuged again at speed 8000 rpm for 5 minutes, after which the supernatant was collected and evaporated to dryness, then 2 ml of methanol was added to it and injected into HPLC (Bhuyian et al., 2015; Huang et al., 2015). A chromatography method was applied to determine the concentration of salicylic in callus using an HPLC device. Type SYKAM-Germany, UV-280nm reagent, and using a C18-ODS column (25 cm*4.6 mm), mobile phase, consisting of ((acetonitrile): MeOH ml (60): 35:5) with a flow rate of 1.0 ml/ Accurate: Sample concentration = area of sample/area of standard solution x concentration of standard solution x number of dilutions.

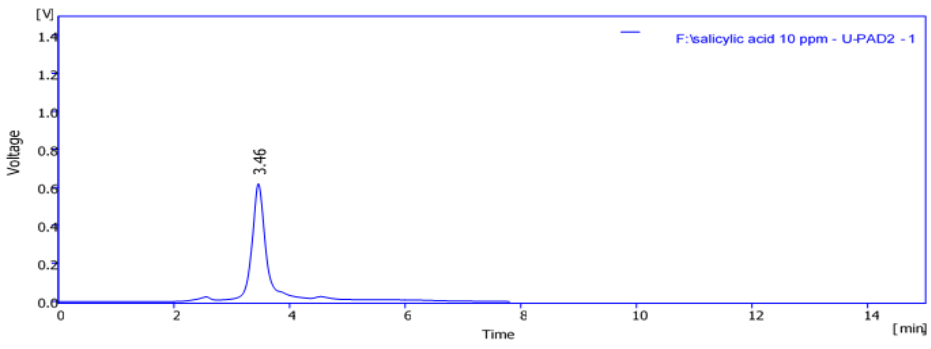

Figure 3-3: Standard curve of salicylic compound in *Calendula arvenesis* callus.

Table 3.2. Result Table (Uncal - F:\salicylic acid 10 ppm - U-PAD2 - 1).

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]	Compound Name
1	3.460	227.567	69.875	100.0	100.0	0.06	
	Total	227.567	69.875	100.0	100.0		

3.10 Extraction of Phenolic Compounds (routine) in the *Calendula arvensis* Plant

According to Radovanovic et al. (2015), the phenolic compound (rutin) was extracted from dry and homogeneous powder of a callus (3 mg) using an ethanol/water solvent (30/70), and the extraction process was carried out using an ultrasonic bath for a period of time.

One hour at room temperature, after filtration 5 ml of the liquid extract was used to determine the extraction yield of solvent evaporation to dryness until the temperature reached 40°C to a constant mass by rotary evaporator and raw and dry were obtained. Dry extracts were stored in containers. Routine quantification was performed by reverse-phase HPLC using a SYKAMN HPLC chromatography system (with UV detector) Chemstation, Zorbax Eclipse Plus-C18-OSD 0.25 cm, 4.6 mm column. The column temperature was 30 °C. The gradient elution method, using eluent A (methanol) and eluent B (1% formic acid in water (v/v)), was performed as follows: 0-4 min, 40% B; 4-10 minutes, 50% B; and the flow rate is 0.7 ml/min. The volume of injected samples was 100 µl and the standard was 100 µl and this was done automatically using the automatic sampler. Spectra were acquired at 280 nm. According to the concentration of the compound according to the equation: Sample concentration = area of sample/area of standard solution x concentration of standard solution x number of dilutions.

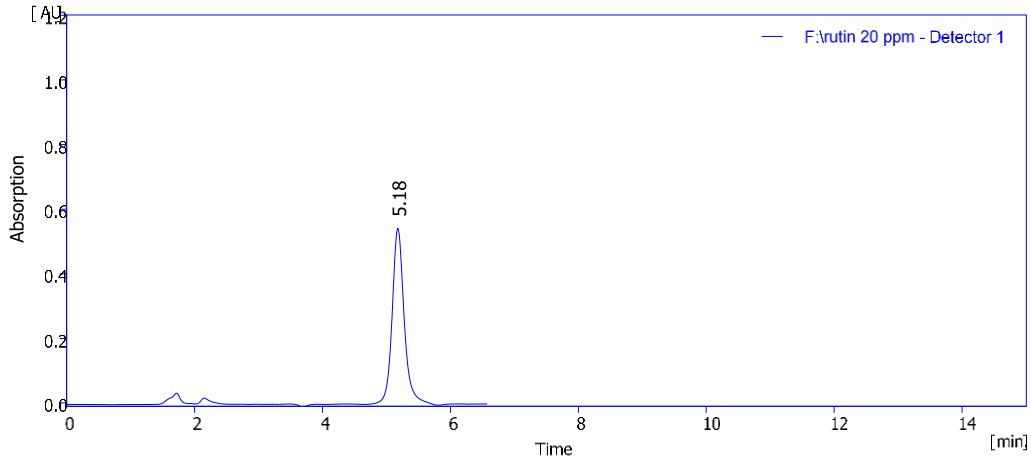

Figure 3-4: Standard curve of phenolic compounds (routine) in the callus of the *Calendula arvenesis* plant.

Table 3-3: Result Table (Uncal - F:\rutin 20 ppm - Detector 1).

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	5.177	313.832	84.475	100.0	100.0	0.07	
	Total	313.832	84.475	100.0	100.0		

4. RESULTS AND DISCUSSION

4.1 Sterilization

Single nodes were taken from plant parts grown in the field and sterilized with three different concentrations of NaClO for different immersion duration, then grown on MS media to test the effect of the sterilized substance and period on the percentage of survival and contamination of plant. As the figure 4.1 shows the highest survival rate was 61% and the least contamination rate was 39% at the concentration of NaClO 10% at the immersion period of 20 minutes.

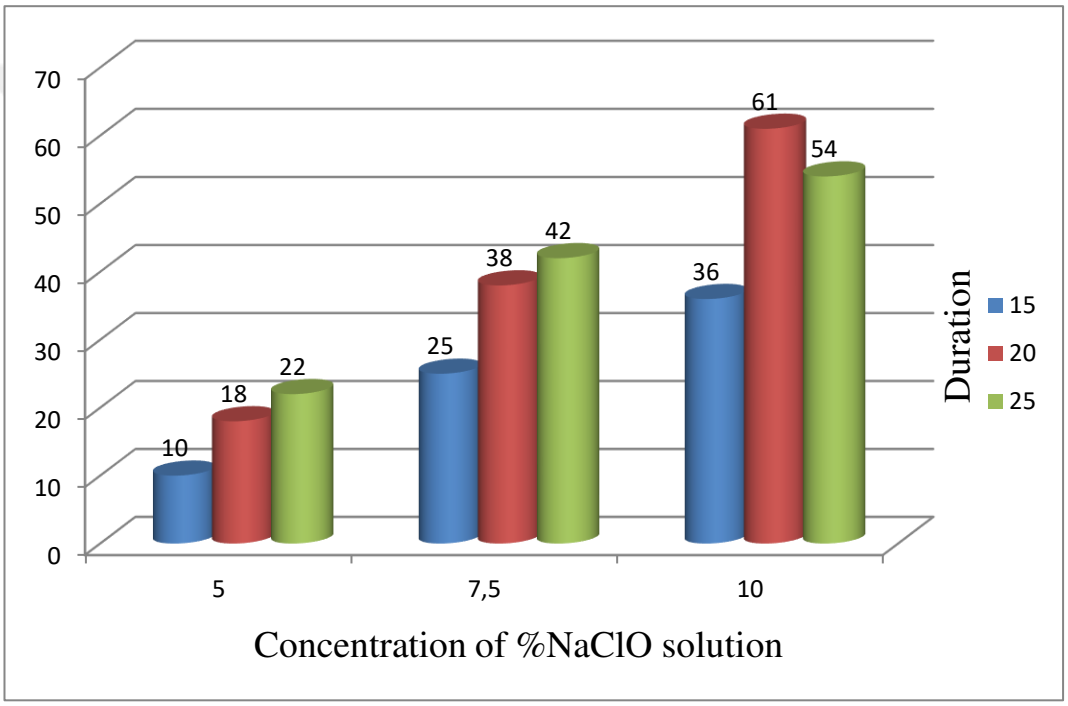

Figure 4.1 Percentage of healthy plant parts (free of contamination).

In order to obtain a good percentage of healthy plant parts, the following protocols were done:

The single nodes of plant parts taken from the field were immersed in ethanol at a concentration of 70% for 30 seconds, then washed with sterile distilled water once, then immersed in three different concentrations in NaCIO solution for a different duration of immersion, and then grown on a MS to see their effect on the percentage of survival and contamination. As figure 4.2 shows that the highest survival rate was 91% when using ethanol at 70% concentration for 30 seconds and using NaCIO solution at 10% concentration for 20 minutes immersion. The table

also shows that the percentage of contamination decreased by using ethanol and increasing the concentrations of NaCIO. The concentration was 10% for 20 minutes as the best concentration for sterilization and was used in all experiments.

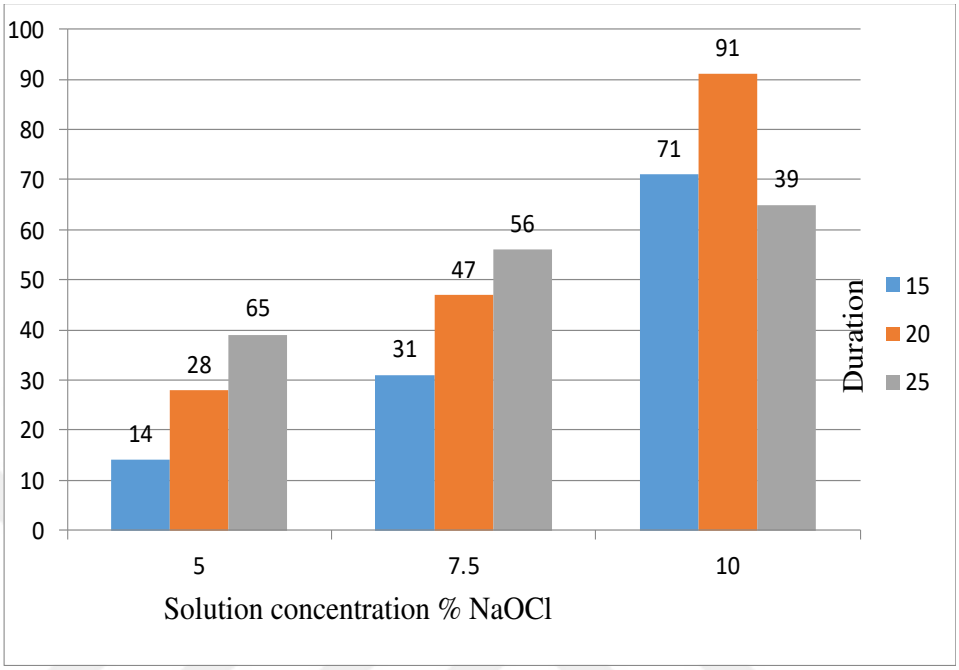

Figure 4.2. Percentage of healthy plant parts (free of contamination).

4.2 Germination Stage

Germination of Calendula arvensis seeds was obtained from tissue mothers when superficially sterilized seeds were grown on MS free medium and germination rate was 100%.

Figure 4.3. A) Seed growth two weeks after planting, B) Seed growth after a month of planting.

4.3 Developmental Stage

Plant parts taken from the field or made up of *in vitro* seed cultures (tissue mothers) were grown on MS medium at different concentrations of KIN and BA to test their effects on the development process.

4.3.1 The Effect of BA on the Development of Nodules Taken from Plants Grown in the Field

Figure 4.4 shows the effect of treatments with different concentrations of BA on the growth of the *Calendula arvensis* node, which had the highest rate of number of 4.7 leaves/part of a plant at a concentration of 0.5 mg/L BA and plant height 1.56 cm at a concentration of 1.0 mg/L BA and above the average number of branches 2.8 branches/plant part at a concentration of 0.5 mg/L after 4 weeks of culturing. Figure 4.4 explains that the addition of BA with different concentrations to the MS medium and after balancing them with the endogenous plant hormones led to an increase in the number of branches (Hopkins and Huner, 1995).

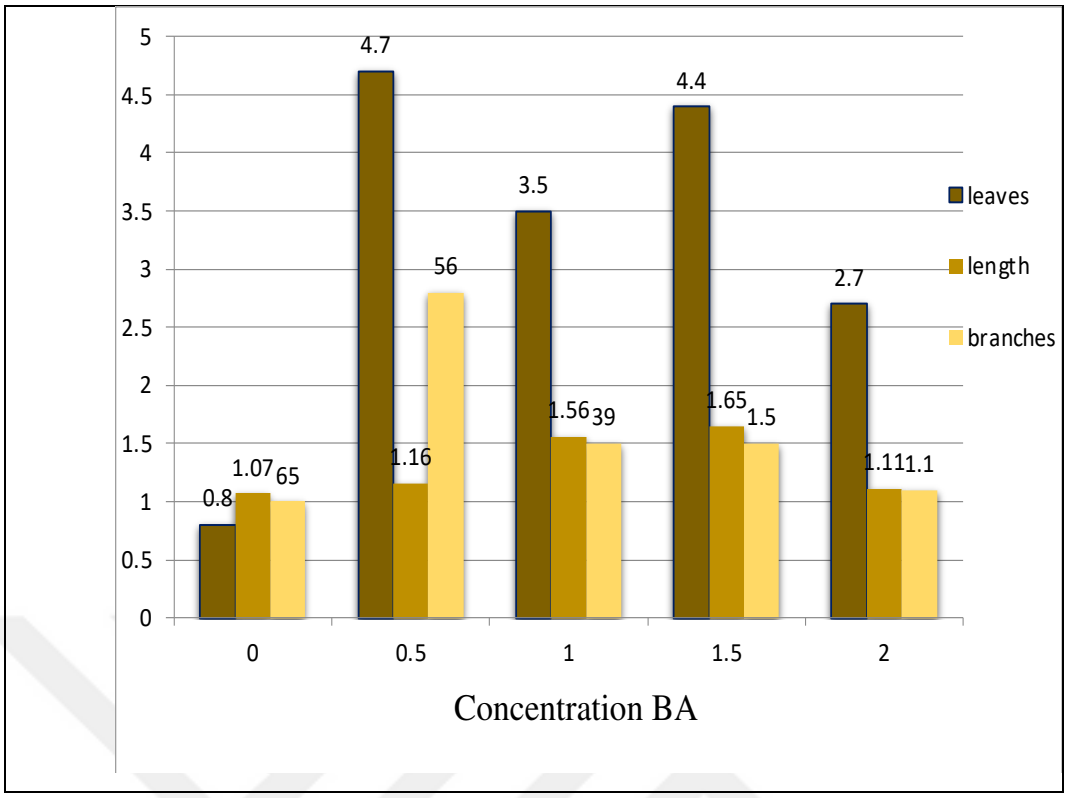

Figure 4.4. The effect of treatments different concentrations of BA on the average number of leaves (leaves/plant part), average length (cm), and average number of branches (branches/plant part).

4.3.2 Effect of BA on the Development of Apex and Nodes Taken from Tissue Mothers

4.3.2.1 Apex

Figure 4.5 shows the effect of different concentrations of BA on the growth of the apical *Calendula arvensis*, where it obtained an average number of leaves 4.6 leaves/plant part at a concentration of 0.5 mg/L BA, and the highest rate of plant was height 5.2 cm at 2 mg/L BA and the highest rate of branching was 2.4 at 0.5 mg/L BA after 4 weeks of planting. The results of the figure 4.5 in growth and development are explained on the basis that BA is one of the most important cytokines that play a major role in controlling the apical dominance and then increasing the number of branches and obtaining the highest values after the result of obtaining a state of balance between the endogenous plant hormones and the used and added growth regulators (Smith, 2000).

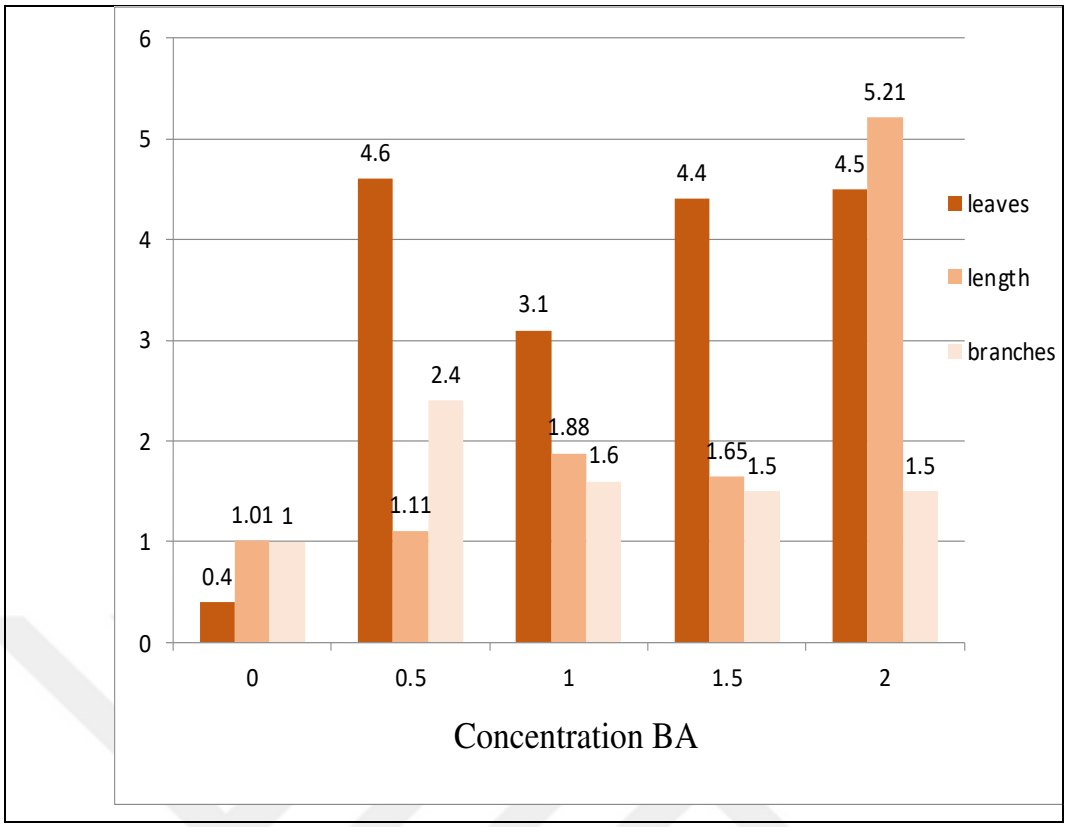

Figure 4.5. The effect of treatments (different concentrations) of BA on the average number of leave (leaves/plant part)s, average length (cm), and the average number of branches (branches/plant part).

4.3.2.2 Nodes

Figure 4.6 shows the effect of different concentrations of BA on the evolution of *Calendula arvensis* nodule, where the highest rate of number of leaves was 7.4 leaves/plant part at concentration 1.0 mg/L BA and the average plant height was 5.68 cm. The highest rate of plant height was at a concentration of mg/L BA, while the highest average number of branches 5.2 branches/part of a plant was at a concentration of 0.5 mg/L BA after 4 weeks of culturing. The results of figure 4.6 explain that the formation of branches and leaves of the cultivated parts in treatment without addition in the emerging stage may be caused by the internal content in the tissues of the plant part of plant hormones (Murashige and Shoog, 1962).

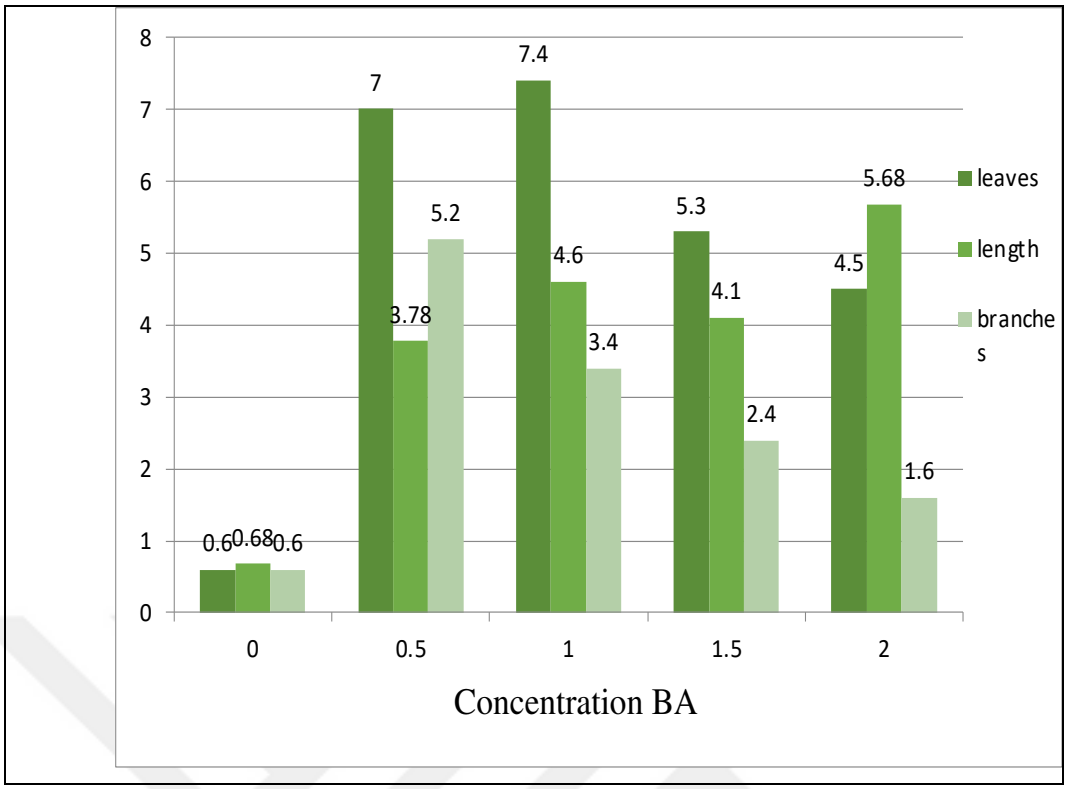

Figure 4.6. The effect of treatments (different concentrations) of BA on the average number of leaves (leaves/plant part), average length(cm), and average number of branches (branches/plant part).

4.3.3 The Effect of KIN on the Development of Apex and Nodes Taken from Tissue Mothers

4.3.3.1 Apex

Figure 4.7 shows the effect of different concentrations of KIN on the growth of the apex of *Calendula arvensis*, where the average number of leaves was 3.0 leaves/plant part at the concentrations 4.0 and 8.0 mg/L KIN and the highest rate of the height of the plant was 2.88 cm at a concentration of 4.0 and 8.0 mg/L KIN and the highest rate of the number of branches 3.1 on the plant part was at a concentration of 2.0 mg/L KIN after 4 weeks of culturing.

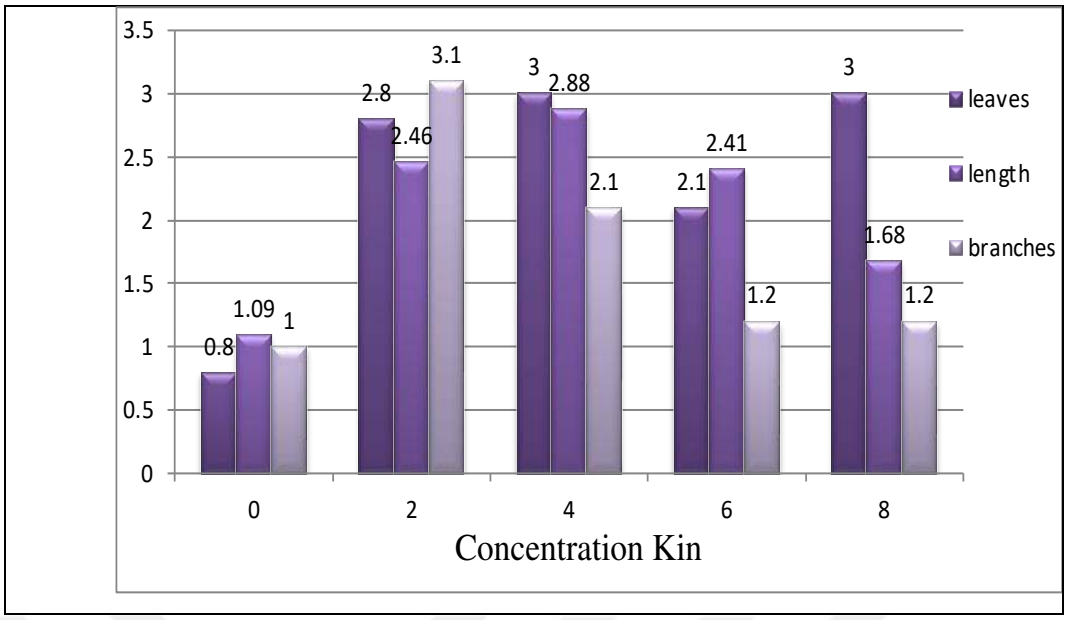

Figure 4.7. The effect of treatments (different concentrations) of kin on the average number of leaves (leaves/plant part), average length(cm), and the average number of branches (branches/plant part).

4.3.3.2 Nodes

Figure 4.8 shows the effect of different concentrations of KIN on the growth of the ganglia of *Calendula arvensis*, where the highest number of leaves was 6.6 leaves/plant part at a concentration of 4.0 mg/L KIN and an average of plant height 4.2 cm at a concentration of 4.0 mg/L KIN, while the highest rate of the number of branches was 4.7 branch/plant part at a concentration of 2.0 mg/L KIN after 4 weeks of planting.

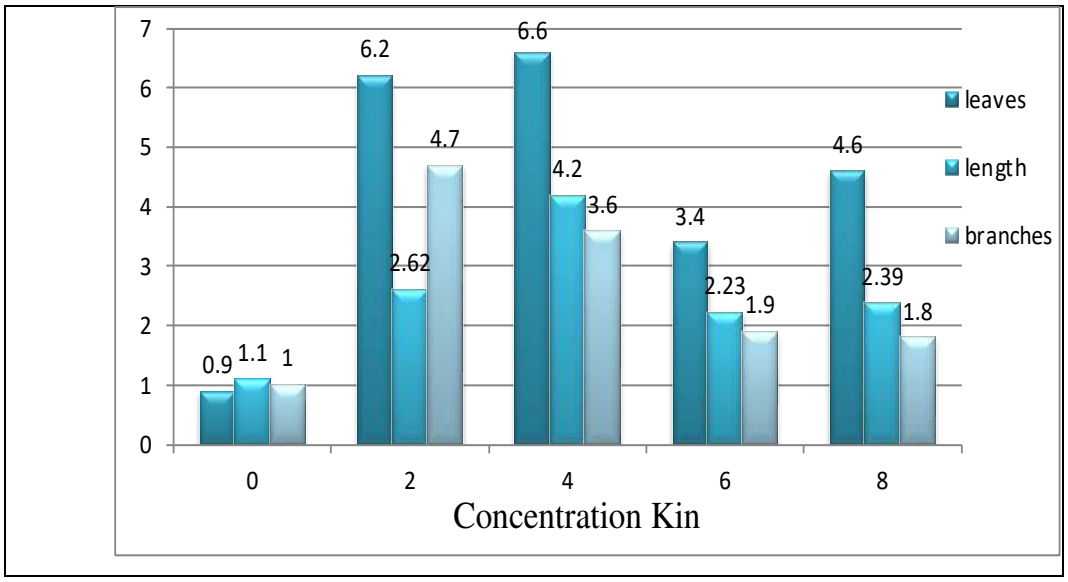

Figure 4.8. The effect of treatments (different concentrations) of KIN on the average number of leaves (leaves/plant part), average length (cm), and the average number of branches (branches/plant part).

The results of figures 4.7 and 4.8 are explained in the emergence of the apex and the node on the basis that KIN is one of the growth regulators that belongs to the group of cytokinins, which in turn plays a major role in eliminating apical dominance and then increasing the number of branches to reach an optimal concentration. An increase in concentration led to a decrease in the number of branches because its effect became in reverse (Smith, 2000).

4.4 Multiplication Phase

4.4.1 Effect of BA on the Doubling of Plant Parts Resulting from the Incipient Stage of Field Plants (non-textile)

Figure 4.9 shows the effect of different concentrations of BA on the doubling of plant parts resulting from the incipient stage. The best dose for number of leaves and plant height was at 1.0 mg/L BA. The values were 5.4 leaves/plant and 2.24 cm, respectively. The highest rate of the number of branches 2.2 branch/plant part at a concentration of 0.5 mg/L BA after two months of planting.

The results of figure 4.9 explain that the reason for using cytokinins in many tissue culture techniques is that they have clear effects on the multiplication of vegetative branches (George et al., 2008). As benzyl adenine is one of the most active cytokinins used in the multiplication of plant parts (Attia and Judua, 1999).

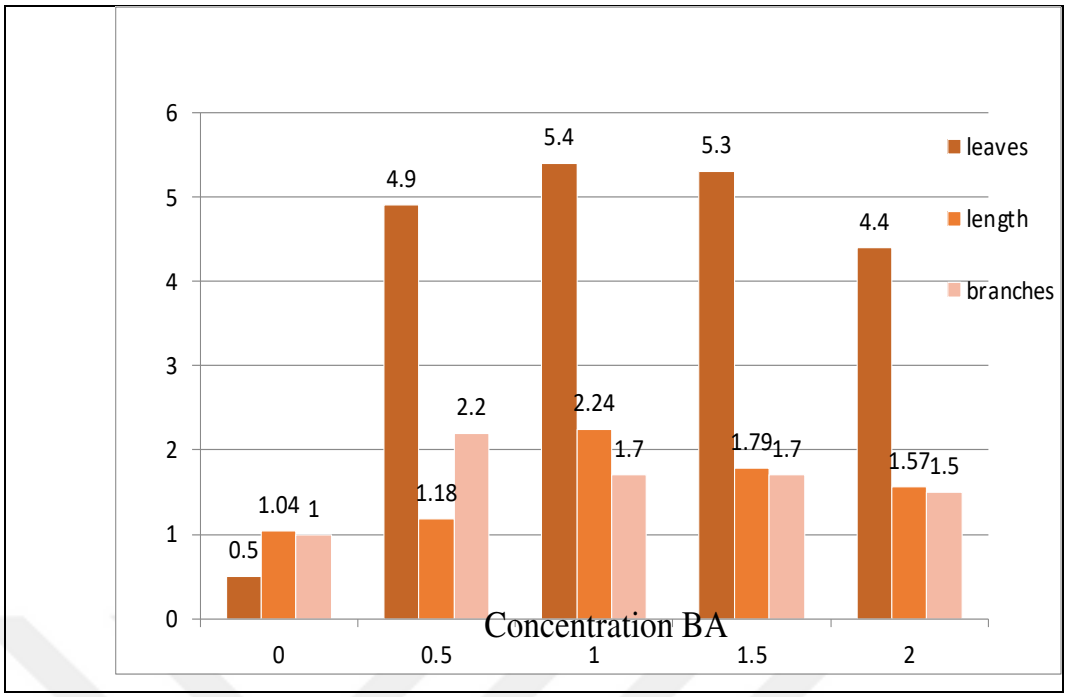

Figure 4.9. The effect of different concentrations of BA on the multiplication of plant parts on the average number of leaves (leaves/plant part), average length(cm), and the average number of branches (branches/plant part).

4.4.2 Effect of BA on the Multiplication of Plant Parts Resulting from the Nascent Stage of Tissue Plants

4.4.2.1 Apex

Figure 4.10 shows the effect of different concentrations of BA on the doubling of the plant parts resulting from the emerging stage, which obtained the highest rate of the number of leaves 7.5 leaves/plant part at a concentration of 0.5 mg/L BA and the highest rate of plant height was 5.14 cm at concentration 0.5 mg/L BA. The highest rate of the number of branches observed 5.5 branch/plant part for a concentration of 0.5 mg/L after two months of planting.

The results of figure 4.10 in the multiplication of the top explains that the increase in the number of leaves and plant length for different BA concentrations treatments leads to the effect of cytokinins in mitosis and cell elongation, which in turn reflects the special growth characteristics as well as the effect of building nucleic acids (Wasfi, 1995).

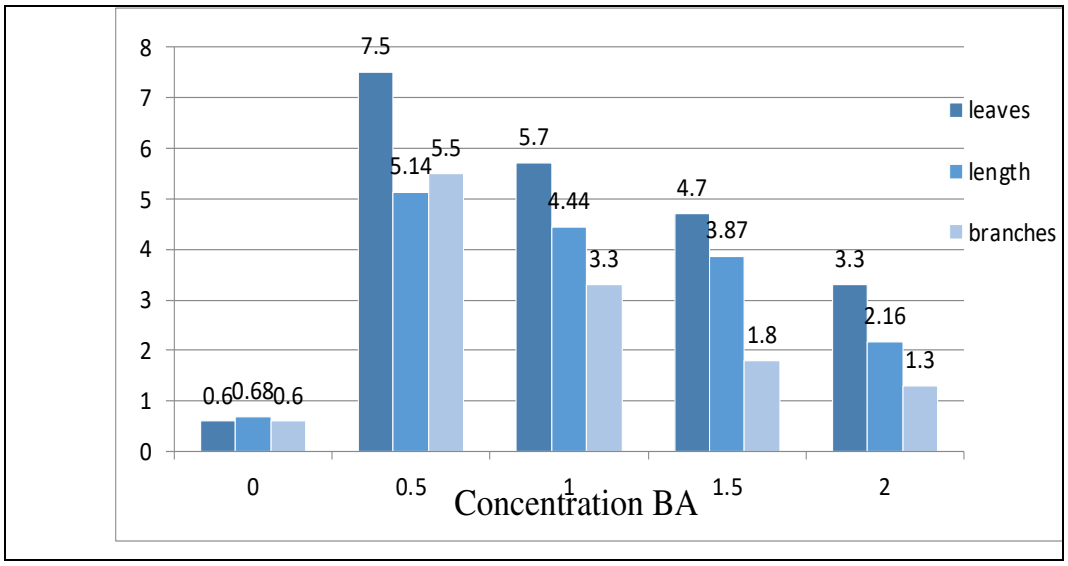

Figure 4.10. The effect of different concentrations of BA on the multiplication of plant parts on the average number of leaves (leaves/plant part), average length(cm), and average number of branches (branches/plant part).

4.4.2.2 Nodes

Figure 4.11 shows the effect of different concentrations of BA on the multiplication of the plant parts resulting from the emerging stage. 0.5 mg/L BA was the best concentration for the number of and value was the 3.7 leaves/plant leaves. 1.53 cm was the highest value for plant height at the concentrations of 1 mg/L and 2 mg/L BA. The highest rate of the number of branches was 2.8 branches/plant part at a concentration of 0.5 mg/L after two months of planting.

The results of the figure 4.11 in the multiplication of the nodes are explained in the light of what was mentioned in the interpretation of the results of the figure 4.10 and this corresponds to (Almemary et al., 2020) when it obtained the best multiplication of the nodes of the dahlia plant on the medium prepared with 0.5 mg/L BA.

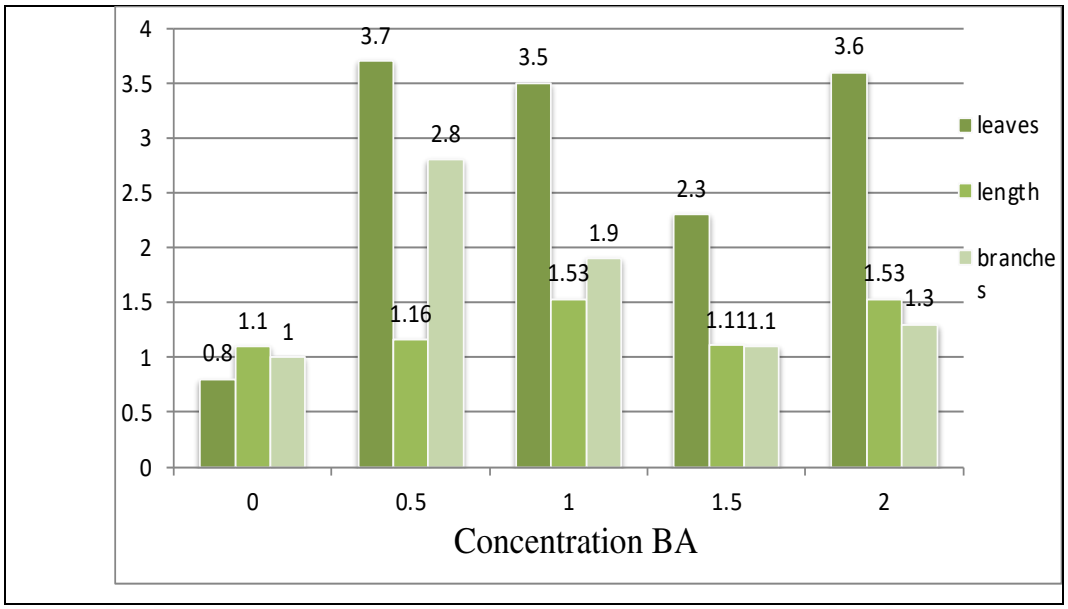

Figure 4.11. The effect of different concentrations of BA on the multiplication of plant parts on the average number of leaves (leaves/plant part), average length(cm), and the average number of branches (branches/plant part).

4.4.3 The Effect of KIN on the Multiplication of Plant Parts Resulting from the Involution Stage of Tissue Plants

4.4.3.1 Apex

Figure 4.12 shows the effect of different concentrations of KIN on the multiplication of the plant parts resulting from the emergence stage, which obtained the highest rate of the number of leaves 8.6 leaves/plant part at a concentration of 2.0 mg/L and the highest rate of plant height was 3.79 cm at the concentration 4.0 mg/L and the highest rate of the number of branches was 4.4 branch/plant part at a concentration of 2.0 mg/L after two months of culturing.

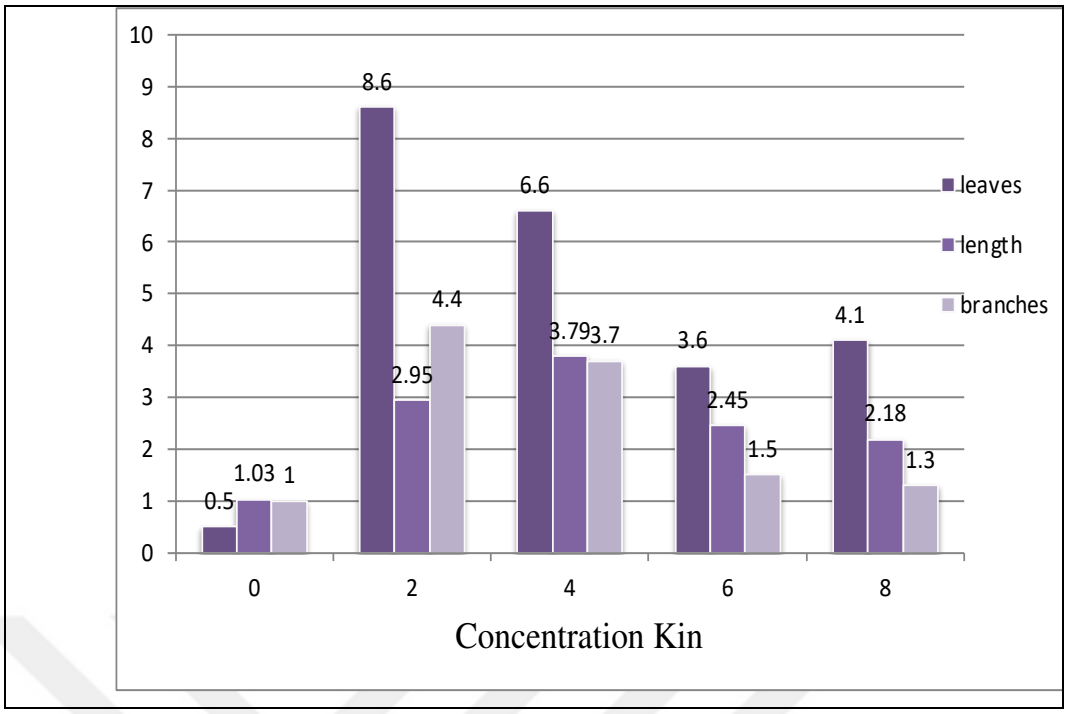

Figure 4.12. The effect of different concentrations of kin on the multiplication of plant parts on the average number of leaves (leaves/plant part), average length(cm), and the average number of branches (branches/plant part).

4.4.3.2 Nodes

Figure 4.13 shows the effect of different concentrations of KIN on the multiplication of the plant parts resulting from the emergence stage, which obtained the highest rate of the number of leaves 6.6 leaves/plant part at a concentration of 4.0 mg/L KIN and the highest rate of plant height was 4.2 cm at a concentration of 4.0 mg/L KIN and the highest rate of the number of branches 4.7 branch/plant part at a concentration of 2.0 mg/L after two months of planting.

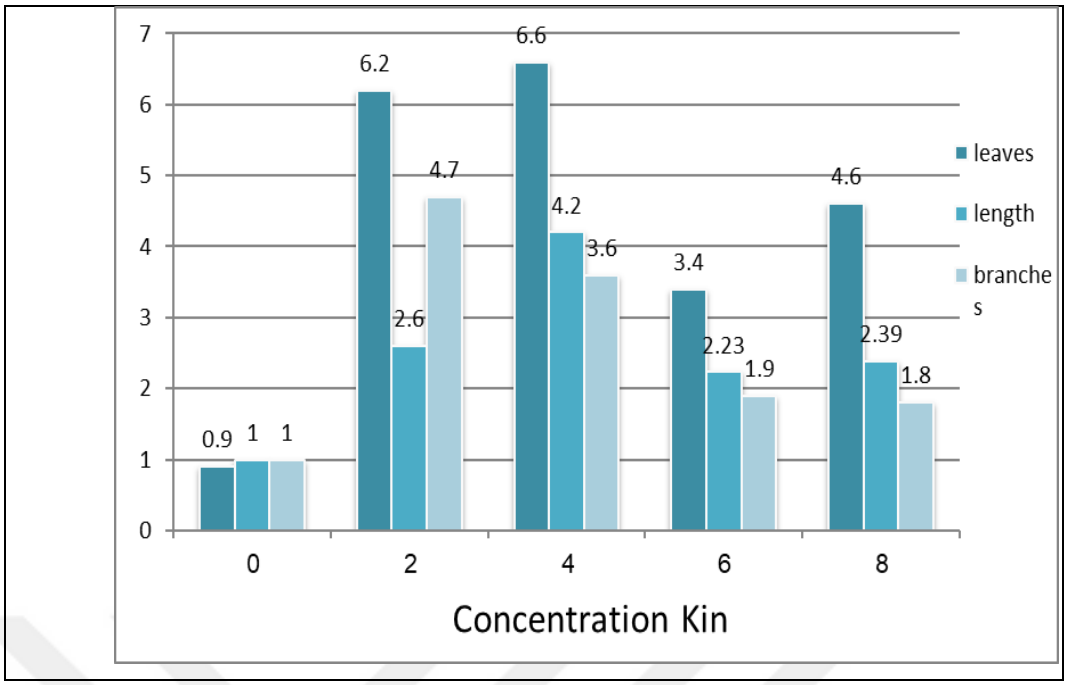

Figure 4.13. The effect of different concentrations of kin on the multiplication of plant parts on the average number of leaves (leaves/plant part), average length(cm), and the average number of branches (branches/plant part).

The results of the figures 4.12 and 4.13 explain that the KIN helps with cell division (Muhammad, 2003), and as a result of its ability to draw nutrients to the growing tops and leaves, it maintains chlorophyll pigment and prevents its loss thus leaves retain their greenness for a long time (Brenner et al., 2005). Regulating the enzymes activity, reducing nitrates and carrying sugars, as well as increasing the number of leaves, number of branches and dry weight of the vegetative group (Sohair et al., 2006).

4.4.4 Effect of Different Concentrations of 2,4-D on the mean Fresh Weight of Callus Induced from *Calendula arvensis* Leaves Taken from Histological Mothers

The significant effect of different concentrations of 2,4-D on callus formation from leaf explant according to average fresh weight of the callus was shown in Figure 4.14. The concentration of 1.5 mg/L 2,4-D was superior by the highest mean fresh weight of callus 1.3230 g which was significantly highest from the concentration of 0.0 mg/L for 2,4-D which gave a 0% generation rate.

The results of figure 4.14 explain the auxins greatly encouraged the growth of the callus, and this may be due to the availability of a certain level of many divisions in the cells of some of them, and they can continue to divide and expand forming the callus tissue (Dodds and Roberts, 1985). And an increase in the wet

weight of the callus is reflections of the changes in the different contents such as on their growth on the medium used, which depends on the growth regulators, carbon source, vitamins etc. and there is an increase in the contents necessary to sustain division and growth in addition to the division of callus cells such as amino acids and proteins (Wiesman et al, 1989). Tissue culture depends on the ability of cells to divide and form new cells, and this is estimated by the increase in cell components and their fresh and dry weight (1989, Abood and Mohamud). This is consistent with Chaitali t al. (2014) that adding to medium at different levels of 2,4-D, where concentrations 1.5 mg/L 2,4-D gave superiority in the soft weight of the callus.

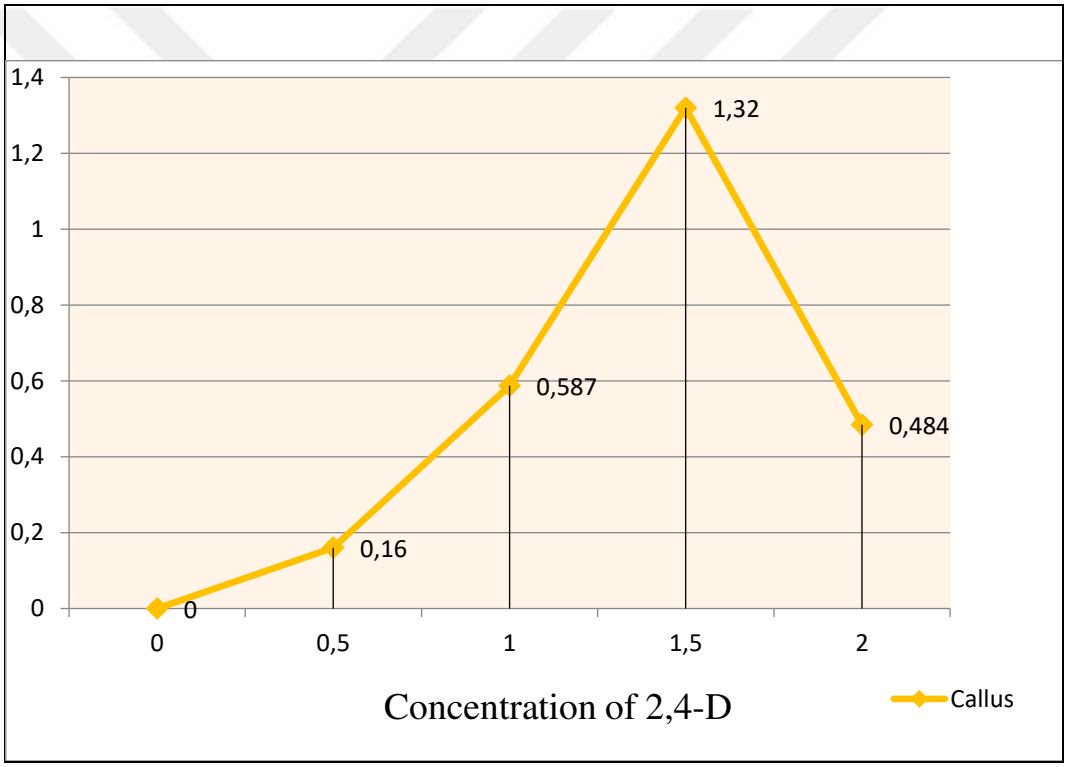

Figure 4.14. Significant difference between the concentrations of 2,4-D in the average fresh weight of callus (g).

Figure 4.15. The size of callus at 1.5 (mg/L) concentration.

4.4.5 Effect of Different Concentrations of 2,4-D on the mean Fresh Weight of Fresh Callus from Leaves of *Calendula arvensis* Plant Taken from Mothers Grown in the Field

The results at the figure 4.16 shows a significant difference between 2,4-D concentrations in the average fresh weight of the callus that was from the leaf explant. The concentration of 1.0 mg/L 2,4-D was superior by the highest mean fresh weight of callus 1.4270 g which was significantly highest from the concentration of 0.0 mg/L for 2,4-D which gave a 0% generation rate.

The results of Figure 4.16 explain that auxins are used in many techniques of plant tissue culture because of their clear and important effect on the growth and induction of callus tissue (George et al, 2008), which confirms that essential factors for the success of the tissue culture process are growth regulators and that the process of callus formation. On the basis of the potential energy of cells and combined with the presence of internal factors related to the genetic composition of plant cells and the level of vitamins and hormones as well as the compatibility of the nutrient medium and growth regulators used in appropriate concentrations that

promote cell division of different explant types may vary their ability depending on their source (Margl et al., 2002).

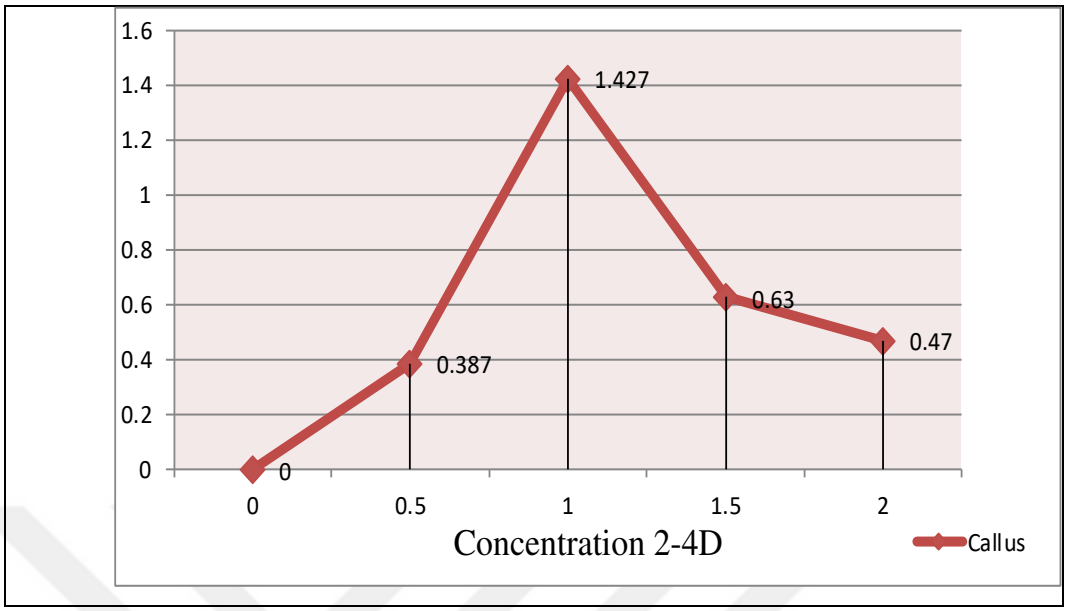

Figure 4.16. Significant difference between the concentrations of 2,4-D in the average fresh weight of the callus(g).

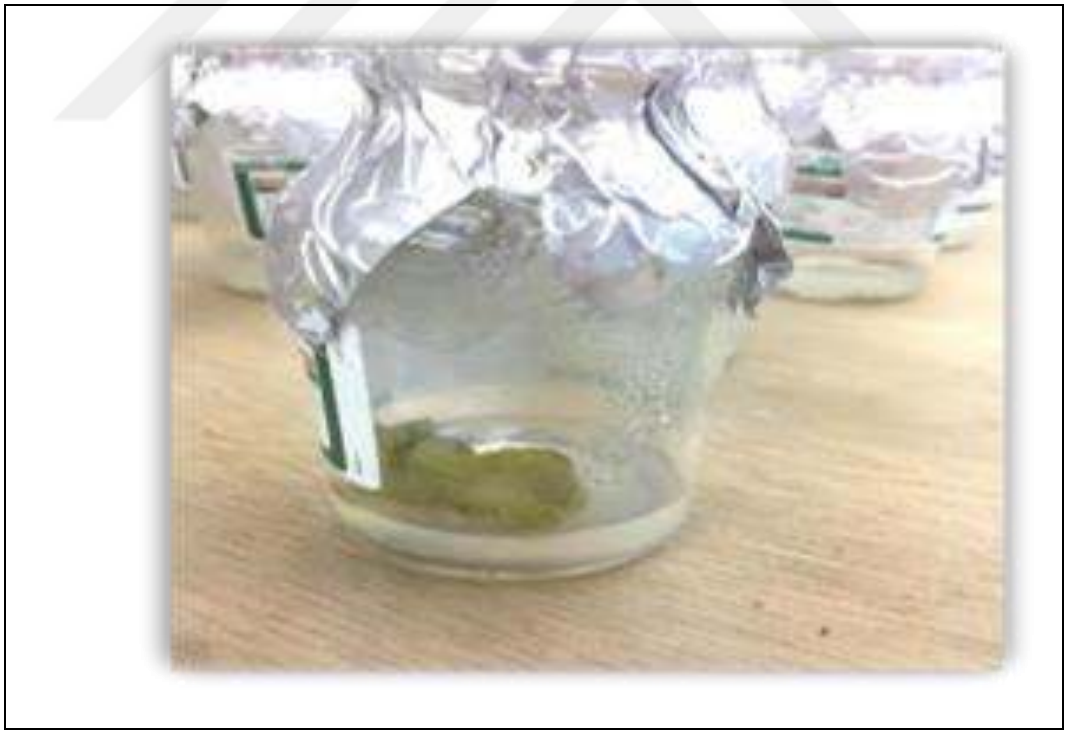

Figure 4.17. The callus size is shown at 1.0 mg/L 2,4-D concentration.

4.4.6 Effect of Adding Brassino acid Catalyst with Different Concentrations on the Average Fresh and Dry Weight of (g) Induced Callus from the Leaves of *Calendula arvensis* Plant

Table 4.1 showed that adding brassino acid with different concentrations to the callus induction medium of *Calendula arvensis* plants led to a decrease in the doubling of the growth of callus compared to the untreated media, which means that the untreated medium gave the highest fresh weight, which amounted to 6.040 g, which differs significantly from the rest of the concentrations. The medium without addition brassino acid to medium had the greatest effect on cell division and gave the highest fresh weight of callus. The fresh weights of the rest of the concentration did not differ significantly from each other and gave a concentration of 0.05 above the dry weight of callus, which amounted to 0.163 g, which was not significantly different from the rest of the concentrations. We concluded from this results, high concentrations of the additive brassino may reduce the callus response in weight gain, which may be attributed to the fact that high concentrations of catalysts work against the action of the catalyst, thus slowing the growth of cells and possibly stopping division process.

Table 4.1. Effect of brassino acid concentrations on average fresh and dry weight (g) of induced callus from ex vivo leaves of *Calendula arvensis* plants after four weeks of cultivation.

Brassino Acid Concentration (mg/L)	Fresh weight (g)	Dry weight (g)
0.0	6.040	0.141
0.05	5.650	0.163
0.1	5.029	0.131
0.2	5.002	0.125

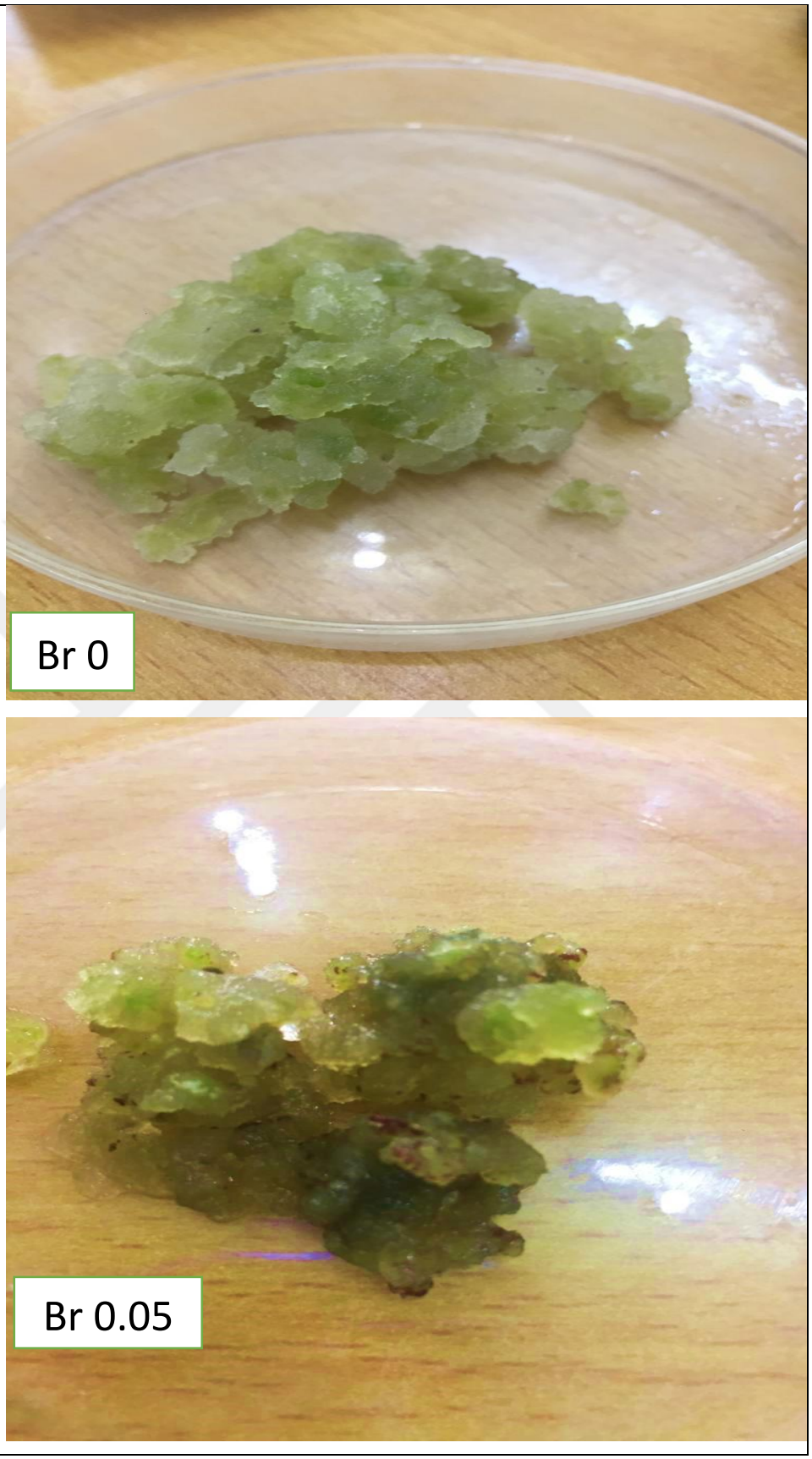

Figure 4.18. Brassino acid concentrations of 0 and 0.05 mg/L.

Figure 4.19. Brassino acid concentrations of 0.1 and 0.2 mg/L

4.4.7 The Catalyst Effect of Brassino Acid on the Production of Active Compounds from *ex vivo* Callus of *Calendula arvensis* Plants

4.4.7.1 Effect of Brassino Acid on the Production of Salicylic Compound

It was observed through the results shown in the table 4.2 that there was an increase in the production of salicylic compound extracted from callus tissue in response to different levels of brassino acid added to the nutrient medium. Where it differed significantly from the treatment without brassino acid, as the value of the compound reached 16.566 (ppm), and the concentration 0.2 was more effective in stimulating the callus tissue in the production of active compounds. That is, brassino stimulated to cell division, development, and growth (Clouse and Zurek, 1991; Oh and Clause, 1998) and activated genes that code for proteins (Felner. 2003), but when used in the right and appropriate doses, it stimulated the plant to grow into the plant growth stage (Swamy and Rao 2008; Hayat, 2010; Eskandari and Eskandari, 2013; Çoban and Baydar, 2017). Brassino has also facilitated photosynthesis by promoting chlorophyll (Duthie et al., 1997); it can alter the content and quality of compounds through its effect on physiological and biochemical pathways and thus alter plant metabolites, but it depends on the plant species (Farooq and Sharma, 1988; Santoro et al., 2013) and its effects on the plant are positive (Naeem et al. 2012).

Table 4.2. The effect of adding different concentrations of brassino acid(mg) on the production of salicylic compound to *Calendula arvensis* plants after four weeks of planting.

Concentrations Br (mg/L)	**Salicylic active compound concentration (ppm)**
0	16.566
0.05	19.733
0.1	31.933
0.2	45.1

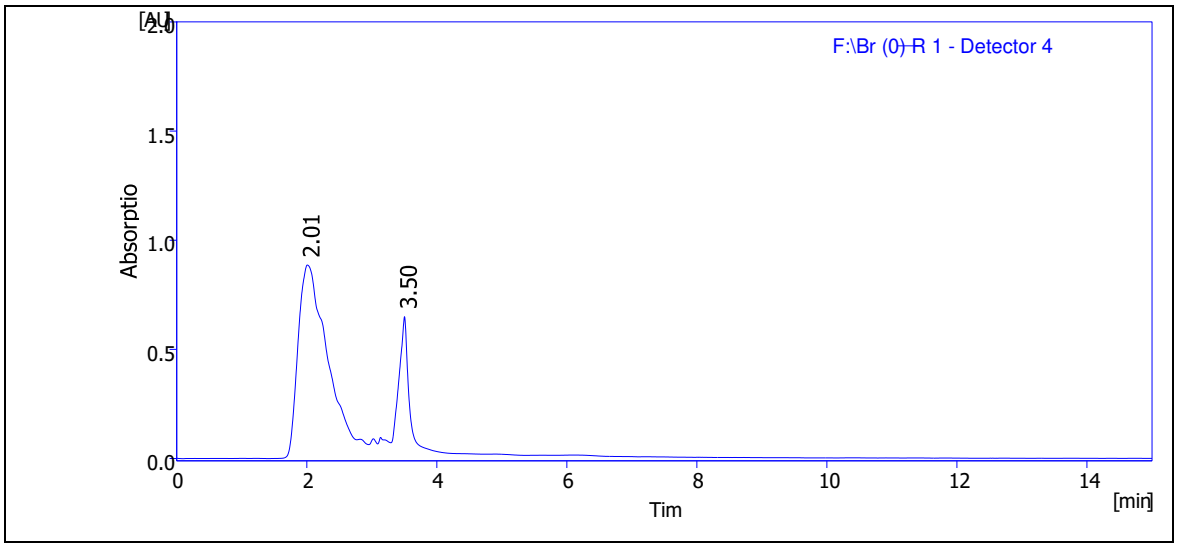

Figure 4.20. Result Table (Uncal - F:\Br (0) R 1 - Detector 4).

Table 4.3. Result Table (Uncal - F:\Br (0) R 1 - Detector 4).

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	2.007	396.145	72.903	48.9	32.2	0.11	
2	3.503	414.376	153.559	51.1	67.8	0.05	
	Total	810.522	226.462	100.0	100.0		

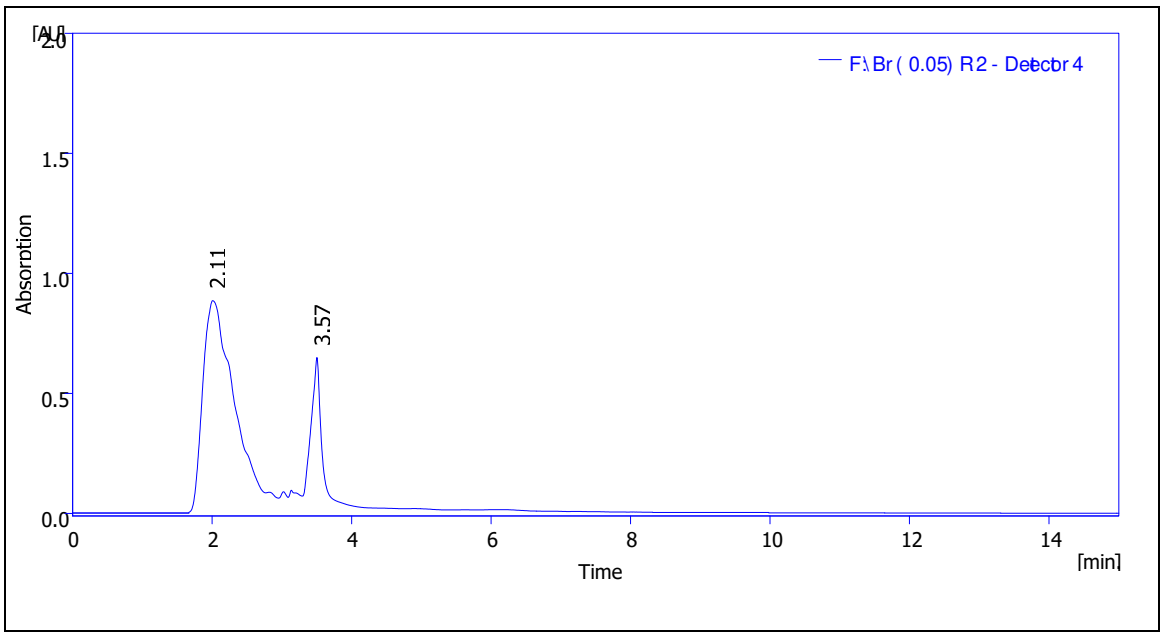

Figure 4.21. Result Table (Uncal - F:\Br (0.05) R 2 - Detector 4).

Table 4.4. Result Table (Uncal - F:\Br (0.05) R 2 - Detector 4).

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W 05 [min]	Compound Name
1	2.110	2847.021	265.231	62.2	42.0	0.19	
2	3.572	1689.625	355.142	37.8	58.0	0.09	
	Total	4536.646	624.356	100.0	100.0		

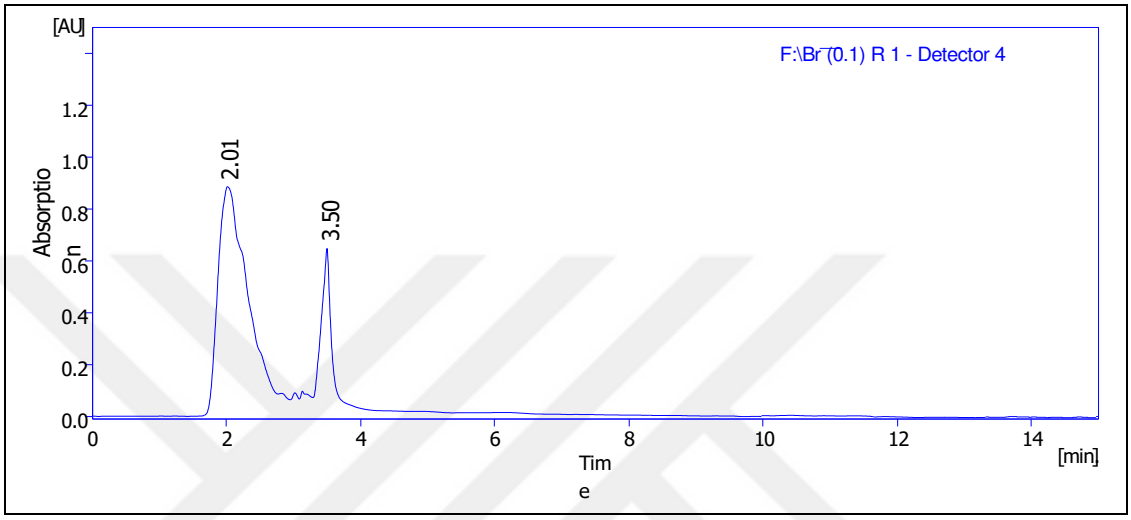

Figure 4.22. Result Table (Uncal - F:\Br (0.1) R 1 - Detector 4).

Table 4.5. Result Table (Uncal - F:\Br (0.1) R 1 - Detector 4).

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W05 [min]	Compound Name
1	2.007	7391.165	443.156	73.6	50.9	0.29	
2	3.503	2651.845	427.815	26.4	49.1	0.11	
	Total	10043.009	870.970	100.0	100.0		

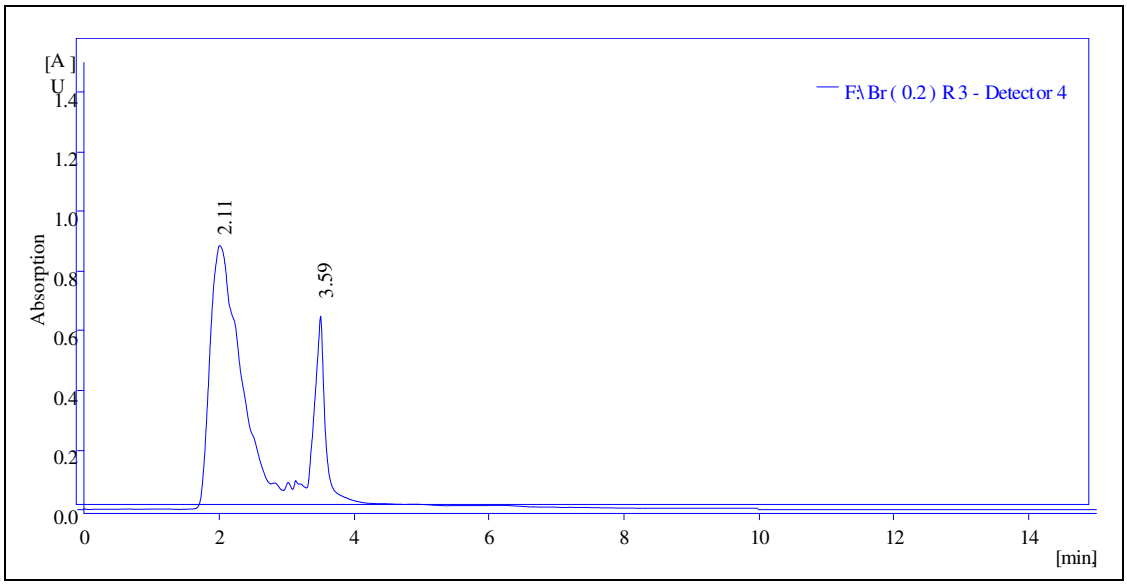

Figure 4.23. Result Table (Uncal - F:\Br (0.2) R 3 - Detector 4).

Table 4.6. Result Table (Uncal - F:\Br (0.2) R 3 - Detector 4).

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W 05 [min]	Compound Name
1	2.110	8823 .214	262.015	59.4	34.5	0.15	
2	3.592	3263.258	451.256	40.6	65.5	0.13	
	Total	12033.058	725.023	100.0	100.0		

4.4.7.2 The Effect of Brassino Acid on the Production of Phenolic Compounds (rutin)

The results presented in Tables 4.7 indicated an increase in morale among the average of the active compounds depending on the concentrations of brassino acid used and added to the nutrient medium. The highest concentration of rutin was reached 80.53 ppm, as it significantly different in the treatment mutrient medium without brassino acid, as the compound value reached 31.233 ppm. Also, adding brassino acid at a concentration of 0.2 to the nutrient medium increased the production of rutin, and this confirms that the acid stimulates gene expression of secondary metabolites and the ability to increase photosynthesis and increase the uptake of water, minerals and nutrients under stress conditions. This compound prevents DNA damage, i.e. prevents geno-toxicity in human lymphocytes in addition to providing protection for a large number of genes against mutations (Duthie et al., 1997), and rutin acts on B-cell hybridoma cells in combination with spleen cells to make an antibody A specific antagonist, which means that the tumor

enhances the cell's ability to grow to constantly secrete immuno-globulins (Alkan, 2004).

Table 4.7. Effect of brassino acid on the production of phenolic compounds (routine) (ppm) for *Calendula arvensis* plants.

Concentration Br(mg/L)	Active Compound Rutine Con (ppm)
0	31.233
0.05	60.1
0.1	74.233
0.2	80.53

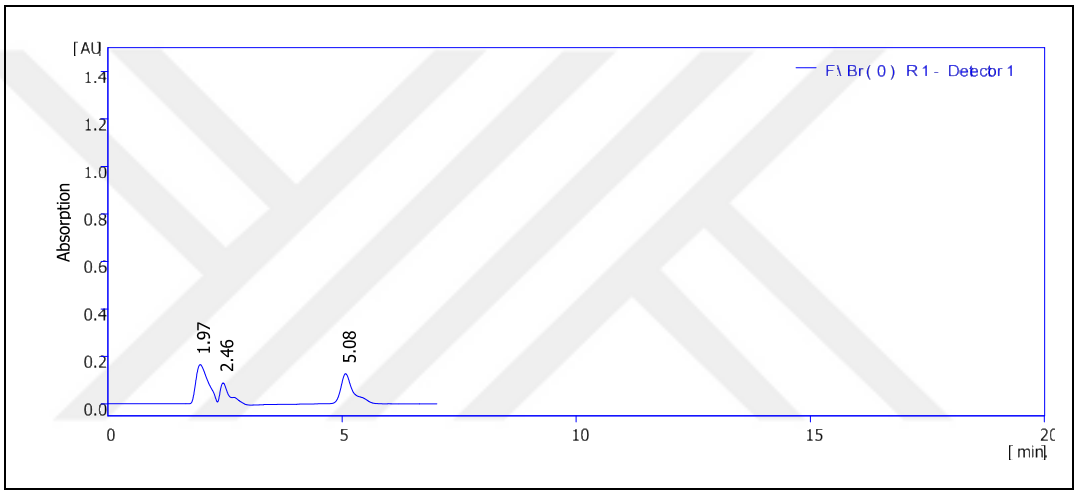

Figure 4.24. Result Table (Uncal - F:\Br (0) R 1 - Detector 1).

Table 4.8. Result Table (Uncal – F:\Br (0) R 1 – Detector 1).

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W 05 [min]	Compound Name
1	1.970	258.878	36.130	38.2	34.1	0.11	
2	2.460	157.079	28.978	23.1	27.3	0.09	
3	5.077	262.608	40.872	38.7	38.6	0.11	
	Total	678.565	105.981	100.0	100.0		

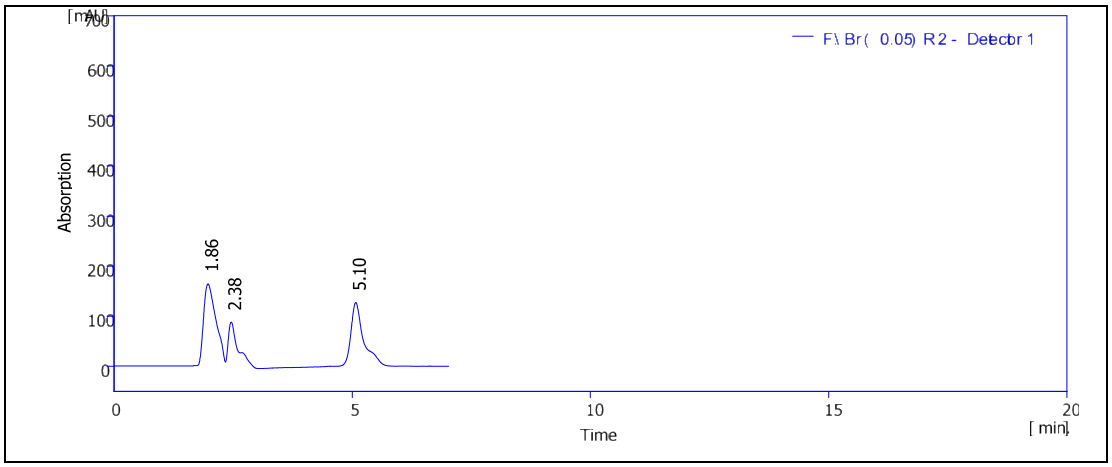

Figure 4.25. Result Table (Uncal - F:\Br (0.05) R 2 - Detector 1).

Table 4.9. Result Table (Uncal – F:\Br (0.05) R 2 – Detector 1).

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W 05 [min]	Compound Name
1	1.860	1621.248	120.145	50.2	43.5	0.23	
2	2.384	621.425	70.655	17.5	23.6	0.14	
3	5.102	1099.325	90.256	33.0	31.8	0.18	
	Total	3341.996	280.215	100.0	100.0		

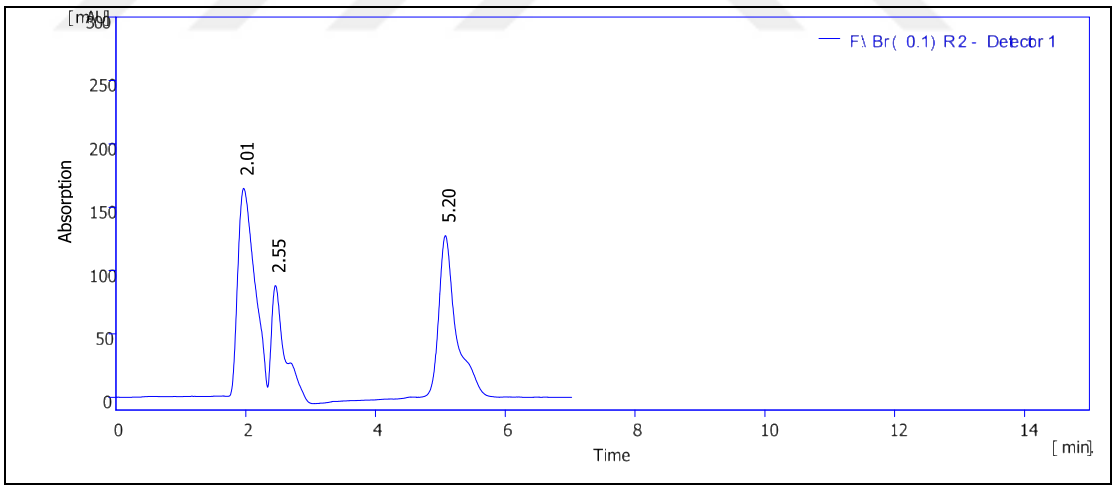

Figure 4.26. Result Table (Uncal - F:\Br (0.1) R 2 - Detector 1).

Table 4.10. Result Table (Uncal – F:\Br (0.1) R 2 – Detector 1).

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W 05 [min]	Compound Name
1	2.011	1425.236	120.589	55.4	48.9	0.22	
2	2.554	535.647	62.658	23.2	26.5	0.15	
3	5.205	1599.258	60.254	22.2	23.9	0.19	
	Total	3560.141	246.258	100.0	100.0		

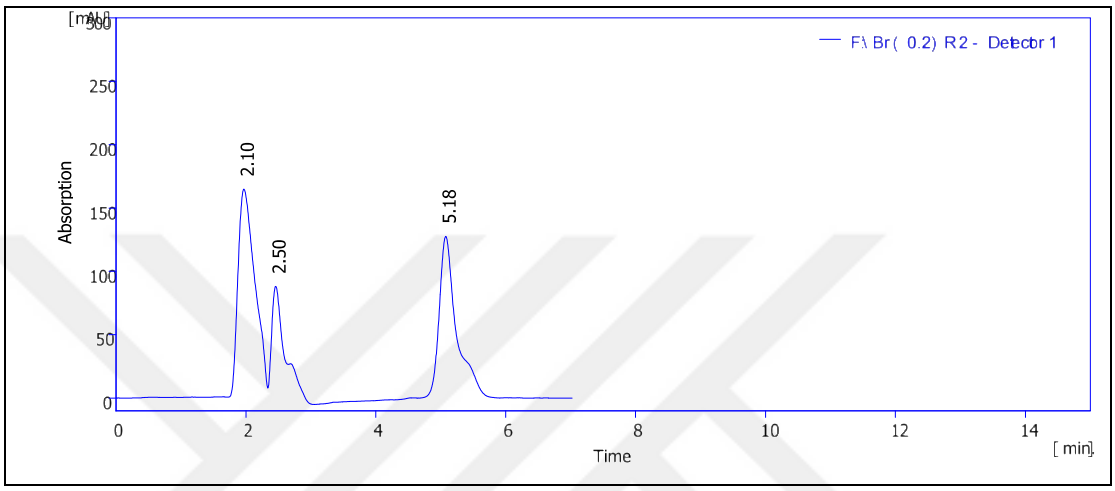

Figure 4.27. Result Table (Uncal - F:\Br (0.2) R 2 - Detector 1).

Table 4.11. Result Table (Uncal – F:\Br (0.2) R 2 – Detector 1).

	Reten. Time [min]	Area [mAU.s]	Height [mAU]	Area [%]	Height [%]	W 05 [min]	Compound Name
1	2.105	1802.258	180.569	30.9	31.5	0.17	
2	2.509	941.325	166.214	21.7	28.5	0.14	
3	5.184	2498.658	196.258	47.2	40.6	0.20	
	Total	5241.256	544.125	100.0	100.0		

5. CONCLUSIONS AND RECOMMENDATIONS

1. The numbers of branches resulting from the cultivation of nodes taken from histological mothers were more than the numbers of branches resulting from the cultivation of nodes taken from plants grown in the field.

2. The cultivation of the leaves resulting from plants grown in the field and tissue plants on the medium treated without addition did not produce any response to the development of callus.

3. The wet weight of the callus formed from planting the leaves obtained from histological mothers was fewer than the wet weight of the callus formed from cultivating the leaves obtained from plants grown in the field on MS medium with the same concentrations.

4. The highest wet weight of callus 1.427 g was obtained after 4 weeks of culturing a part of the resulting leaf from plants grown in the field on medium supplemented with 1.0)mg/L 2,4-D.

5. The average number of branches resulting from planting nodes on MS medium supplemented with different concentrations of BA was greater than the numbers of branches resulting from planting nodes on MS medium supplemented with different concentrations of KIN.

6. Leaves taken from textile plants were better at producing callus than leaves taken from plants grown in the field.

7. The development of callus from planting part of the leaf taken from tissue or non-textile plants in the field.

8. The sterilization method with sodium hypochlorite (Fez commercial) at concentration (10%) and for 20 minutes was determined as the best way to sterilize the seeds of the *Calendula arvensis* plant.

9. There was a gradual increase in the content of secondary compounds with the increase in concentrations of brassino, as the concentrations of 0.2 mg/L and 0.1 mg/L recorded the highest amount of salicylic and rutin compounds sequentially, while the lowest amount was in the treatment (without the addition).

It is possible to include the medium with initiators and other growth regulators and chemical stimuli at various other levels. These stimuli have a role in the accumulation of active compounds determining the appropriate concentration that is reflected negatively or positively in the accumulation of those compounds.

6. REFERENCES

"APA citation system was used in this thesis."

Abed Elmaksood, W. M., Ebad, F. A., & Bosila, H. A. (2016). In vitro Propagation of the Endangered Medicinal Plant *Hyoscyamus muticus* L. (Egyptian Henbane). *Journal of Applied Environmental and Biological Sciences*, 6 (4), 25-34.

Abraham, M. (2008). Studies On Ephedrine Production in Callus Cultures Derived from Colchicine Treated Seeds snd Seedling of Sida Species. *University of Kerala* PhD Thesis, India

Abu –Qaoud, H., Abu-Rayya, A., & Yaish, S. (2010). *In vitro* Regenerationand Somaclonal Variation of Petunia hybrida. *Journal of Fruit and Ornamental Plant Research*, 18 (1): 71-81.

Abudunia, A., Ansar, M., Taoufik, J., Ramli, Y., Essassi, M., Ibrahimi, A., & Khedid, K. (2014). Evaluation of Antibacterial Activity of Extracts from *Calendula aventis* Flowers. *Journal of Chemical and Pharmaceutical Research*, 6(8):156 -161.

Abutaha, N., Nasr, F. A., Mohammed, A., Semlali, A., Al-Mekhlafi, F. A., & Wadaan, M. A. (2019) *Calendula arvensis* L. as an anti-cancer agent against breast cancer cell lines. Molecular *Biology Reports,* 46:2187–2196.

Ahmed, A. A., Jakupovic, J., & Mabry, T. J. (1993). Sesquiterpene Glycosides from *Calendula arvensis*. *J. Nat. Prod*, 56 (10): 1821-1824.

Al-Abbasi, I. N., & Kassab-Bashi, B. Z. (2013). Micropropagation of Purpurea *Digitalis purpurea* L. *Journal of Rafidain Agriculture,* 41 (4): 85-95.

Al-Akedi, H. N. A., & Kassab Bashi, B. Z. (2018). Duplication of the Knot, Development and Differentiation of the Callus of the Plant *Atropa belladonna* L. *Tikrit Journal of Agricultural Sciences*, 18(1): 99-112.

Al-Badrany, K. T. S. (2014). The Use of Tissue Culture Technology in Propagation of Chrysanthemum morifolium. University of Mosul, College of Agriculture and Forestry Master's Thesis, Mosul.

Al-Dabagh, F. M. K., & Salih, M. I. (2020). Chia Seed as a Source of *In vitro* Establishment of *Salvia hispanica* L. *Plants. Iraqi Journal of Agricultural Sciences*, 51 (3): 976-981.

Al-Fatlawi, Z. H. J. (2014). Using the Plant Tissue Culture Technique to Obtain the Spilanthol Compound from *Spilanthes Acmella* (L.) Murr. College of Science for Girls. Baghdad University Master's Thesis, Iraq.

Al-Hadidi, M. A. H. (2002). Experiments in Tissue Culture. *Dar Al-Fikr for printing, publishing and distribution*, Amman, Jordan.

Al-Hasani, A. Z. J. (2013). Vegetative Propagation of *Spilanthes acmella* (L.) Murr. Outside the living body, College of Science for Girls, University of Baghdad Master's Thesis, The Republic of Iraq.

Al-Hayani, S. A. M. A. (2014). Response of Dahlia variabilis L. cultivar, Arizona to growth and propagation in field and ex vivo conditions Master's thesis, College of Agriculture, University of Diyala.Iraq.

Alkan, S. (2004). Monoclonal Antibodies: the Story of a Discovery that Revolu- tionized Science and Medicine. *Nat Rev Immunol*, 4: 153–156.

AL-Khafaji, M. A. (2014). Plant Growth Regulagtors, *Application and utilizations in Horticlture. University house for printing, publishing and translation. University of Baghdad*- Iraq.348.

Al-Kinani, F. R. (1987). Tissue and Plant Cell Culture. *Directorate of Books and Printing House / University of Mosul Iraq*.

Al-Mamari, A. M. S. (2014). Development and Differentiation of Callus from Some Parts of the Plant Catharanthus Roseus and Estimation of its Content of Some Alkaloids, College of Agriculture and Forestry PhD Thesis, University of Mosul.

Almemary, A. M. S., Faizy, W. S., & Kassab Bashi, B. Z. (2020). *In vitro* Multiplication of Shoot tip and Nodes in Dahlia hybrida. *International Journal of Agricultural and Statistical Sciences*, 16 (1): 259-263.

Al-Mukhtar, H., Mahmod, S. A., & Abd, A. M. (2019). Effect of growth regulator in micropropagation of Catharanthus roses *in vitro*. Journal of Physics, Conference Series, 1294 (9): 1-9.

Al-Musawi, Z. J. N. (2017). The effect of some physical and chemical factors on the production of secondary materials from the plant Tanacetum parthenium L. cultivated in the field and in the laboratory, College of agricultural engineering sciences university of Baghdad PhD thesis, Iraq.

Al-Rifai, A. T., & al-Shobaki, S.A. (2002). 21st century techniques for plant improvement using tissue culture. *Arab Thought House for Printing and Publishing,* Egypt.

Alvarez, D. F., Gjerde, E. A., & Townsley, M. I. (2003). Role of EETs in regulation of endothelial permeability in rat lung. *Am J Physiol Lung Cell Mol Physiol*, 286: 445–451.

Al-Zuhairi, I. M. A. (2016). Effect of some chemicals on the growth of Ain al-Bazon plant and its production of indole alkaloids inside and outside the body, Diyala University, Faculty of Agriculture master's thesis, Iraq.

Aminnejad, M., Hosseini, B., & Qaderi, A. (2015). Effect of plant growth regulators and explant types on in vitro direct plant regeneration of *Hyoscyamus reticulatus* L. *International Journal of Advanced Research*, 3 (3): 457-462.

Arif, T. (2015). Salicylic acid as a peeling agent: A comprehensive review. *Clin. Cosmet. Investig. Dermatol,* 8: 455–461.

Arora, D., Rani, A., & Sharma, A. (2013). A review on phytochemistry and ethnopharmacological aspects of genus Calendula. *Pharmacogn Rev*, 7(14):179.

Asha, A., & Lingakumar, K. (2015). Effect of 24-epibrassinolide spray on vegetative growth, pigment composition and biochemical constituents of Vignamungo (L.) Hepper (Blackgram). *J.Global Biosci*, 4(4): 2007-2012.

Attia, H. J., & Judua, K. A. (1999). Theoretical and applied plant growth regulators. *Ministry of Higher Education and Scientific Research, National Library Press* / Baghdad - Iraq.

Bajguz, A., & Piotrowska-Niczyporuk, A. (2014). Interactive ef-fect of brassinosteroids and cytokinins on growth, chloro- phyll, monosaccharide and protein content in the greenalga Chlorella vulgaris (*Trebouxiophyceae*). *Plant Physiol. Biochem*, 80: 176–83.

Bashi, A. A. A. (2020). Micropropagation of Petunia hybrida plant. University of Mosul College of Agriculture and Forestry Master Thesis, Iraq.

Basu, A., & Kumar, G. S. (2014). Interaction of the dietary pigment curcumin with hemoglobin: energetics of the complexation. *Food and function.J. Royal Society of Chemistry,* 5: 1949–1955.

Beecher, G. R., Warden, B. A., & Merken, H. (1999). Analysis of tea polyphenols. *Proc Soc Exp Biol Med*, 220: 267–270.

Bharathi, B., Sharmiladovi, R., & Daniel, G. S. (2010). Studies on antibacterial activity and phytochemical analys8is of Datura metel L. against bacterial pathogens associated with AIDS. *Advanced Biotech,* 10(3): 21-25.

Bhuyian, H. U., Rashid, H., Ariful lslam, A. F. M., & Tareque, M. D. I. (2015). Development and Validation of Method for Determination of Clobetasol Propionate and Salicylic Acid form Pharmaceutical Dosage Form by HPLC. *J. of Pharmaceutical Research* 7(5), 374 -385.

Bravo, L. (1998). Polyphenols: chemistry, dietary sources, metabolism and nutritional significance. *Nutr Rev*, 56, 317–333.

Brenner, W. G., Romanov, G. A., Kollmeri, B., & Schmulling, T. (2005). Immediate-early and delayed cytokinin response genes of Arabidopsis thaliana identified by genome-wide expression profiling reveal novel cytokinin sensitive processes and suggest cytokinin action through transcription alcascades. *Plant J.,* 44, 314-333.

Buchner, A. (1828). Ueber das rigatellische fiebermittel und über eine in der weidenrinde entdeckte alcaloidische substanz. *Repert. pharm,* 29, 405–420.

Buchner, N., Malti, S., & Preeti, K. (2014). Callus induction and plant regeneration from leaf explants of Spilanthes acmella murr- an endangered medicinal plant. *Annal of biological research*, 5(9), 66 -71.

Chaitali, N., Malti, S., & Preeti, K. (2014). Callus induction and plant regeneration from leaf explants of Spilanthes acmella murr, *Annal of biological research* 5(9), 66-71.

Cheynier, V. (2012). Phenolic compounds: From plants to foods. *Phytochem. rev,* 11, 153–177.

Clouse, S. D., & Zurek, D. (1991). Molecular analysis of brassinolide action in plant growth and development. In Brassinosteroids; Chemistry, Bioactivity and Applications, ACS Symposium Series 474. In: Cutler HG, Yokota T, Adam G (eds). pp. 122-140. American Chemical Society, Washington D.C.

Çoban, Ö., & Baydar, N. G. (2017). Brassinosteroid modifies growth and essential oil production in peppermint (*Menthapiperita* L.). J. *plant growth regul*, 36, 43-49.

Coste, H. (1937). Flore descriptive et illustrée de la France, de la Corse et des contrées limitrophes (Tome 1), *Librairie des Sciences et des Arts*, Paris, 289-305.

Dachineni, R., Kumar, D. R., Callegari, E., Kesharwani, S. S., Sankaranarayanan, R., Seefeldt, T.; Tummala, H. & Bhat, G. J. (2017). Salicylic acid metabolites and derivatives inhibit CDK activity: Novel insights into aspirin's chemopreventive effects against colorectalcancer. *Int. j. oncol,* 51: 1661–1673.

Davis, J. M., Murphy, E. A., McClellan, J. L., Carmichael, M. D., & Gangemi, J. D. (2008). Quercetin reduces susceptibility to influenza infection following stressful exercise. *Am j physiol regul Integr compphysiol,* 295, 505–509.

De Tommasi, N., Pizza, C., Conti, C., Orsi, N., & Stein, M. L. (1990). Structure and in vitro antiviral activity of sesquiterpeme glycosides from Calendula arvensis. *J, nat. prod*, 53(4),830-835.

Del Valle, E. M. M. (2004). Cyclodextrins and their uses: A review. *Process biochemistry*, 39, 1033–1046.

Dello loio, R., Linhares, F. S., Scacchi, E., Casamitjana-Martinez, E., Heidstra, R., Costantino, P., & Sabatini, S. (2007). Cytokininus determine Arabidopsis thaliana root meristem size by controlling cell differentiation. *Curr. biol*. 17(8), 678-682.

Dempsey, D. A., Vlot, A.C., Wildermuth, M.C., & Klessig, D. F. (2011). Salicylic acid biosynthesis and metabolism. *Arab. book,* 9, e0156.

Dempsey, D. A., & Klessig, D. F. (2017). How does the multifaceted plant hormone salicylic acid combat disease in plants and are similar mechanisms utilized in humans? *BMC biol,* 15, 23.

Dhanarasu, S. (2012). Chromatography and Its Applications Edited ISBN 978- 953-51-0357-8 Hard cover, Publisher InTech, 224 pages

Dodds, J. H., & Roberts. j. W. (1999). *Experiments in plant tissue culture*. Third Edition.Cambridge Univ. Press. London, New york.

Duthie, S. J., Collins, A. R., Duthie, G. G., & Dobson, V. L. (1997). Quercetin and myricetin protect against hydrogen peroxide-induced DNA damage (strand breaks and oxidised pyrimidines) in human lymphocytes. *Mutat res,* 393, 223–231.

Elshorbagy, M. I., Ibrahim, S. M., Abo El Seoud, K. A., & Abd El Maksoud, A.I. (2018). Tropane alkaloids production from callus culture of *Atropa belladonna* L. as affected by elicitors and precursor feeding. *International research journal of pharmacy*, 9 (7), 116-125.

Ercetin, T., Senol, F. S., Orhan, I. E., Toker, G. (2012). Comparative assessment of antioxidant and cholinesterase inhibitory properties of the marigold extracts from *Calendula arvensis* L. and Calendula officinalis L. *Ind Crops Prod*, 36, 203–208.

Erlund, I., Kosonen, T., Alfthan, G. (2000). Pharmacokinetics of quercetin from quercetin aglycone and rutin in healthy volunteers. *Eur J Clin Pharmacol,* 56: 545–553.

Eskandari, M., & Eskandari, A. (2013). Effects of 28-homobrassinolide on growth, photosynthesis and essential oil content of Saturejakhuzestanica. *Int. j. plant physiol. Biochem*, 5(3), 36-41.

Fariduddin, Q., Yusuf, M., Chalkoo, S., Hayat, S., & Ahmad, A. (2011). 28-homobrassinolide improves growth and photosynthesis in Cucumis sativus L. through an enhanced antioxidant system in the presence of chilling stress. *Photosynthetica,* 49: 55-64.

Farooq, H.A., & Sharma, S. (1988). Effect of growth retardants on growth and essential oil content in Japanese mint. *Plant Growth Regul*, 7(1), 39-45.

Felner, M. (2003). Recent progress in brassinosteroid research: Hormone perception and signaltransduction. In: Hayat S. and Ahmad A (eds) Brassinosteroids: Bioactivity and Crop Productivity. Kluwer- Dordrecht, p 69-86.

Formica, J. V., & Regelson, W. (1995). Review of the biology of quercetin and related bioflavonoids. *Food Chem Toxicol*, 33: 1061–1080.

Frankel, E. N., & Meyer, A. S. (2000). The problems of using one-dimensional methods to evaluate multifunctional food and 365 biological antioxidants. *Journal of the science of food and agriculture*, 80, 1925-1941.

Gamborg, O. L. (2002). Plant tissue culture. Biotechnology milestones. *In vitro cell dev. biol. plant,* 38, 84-92.

Geetha, V.S., Viswanathan, S., & Kameswaran, L. (1981). Comparison of total alka-loids of Thylophora indica and disodium cromoglycate on mast cell sta- bilization. *Indian j. pharmacol*, 13, 199–201.

George, E. F., Hall, M. A., & Deklerk, G. J. (2008). Plant propagation by tissue culture. Volume 1. The Background, 3rd (ed.), Edition, Published by Springer, Dordercht. the Netherlands. 1-479.

Gomes, M. M. A. (2011). Physiological effects related to brassinosteroid application in plants. - In: Hayat, S., Ahmad, A. (ed.): Brassinosteroids: a Class of Plant Hormones. Pp. 193-242. Springer, Dordrecht - Heidelberg - London - New York.

Grobelak, A., & Hiller, J. (2017). Bacterial siderophores promote plant growth: Screening of catechol and hydroxamate siderophores. *Int. j. phytoremediation*, 19, 825–833.

Grzegorczyk, I., Bilichowski, I., Olasik, E., & Wysokinsk. H., (2005). *In vitro* culture of Salvia officinalis as source of antioxidant compounds. Acta. Soci. Botanicorum Poloniae, 74(1), 17-21.

Gu, X. Y., Liu, Y., & Liu, L. J. (2020). Progress on the biosynthesis and signal transduction of phytohormone salicylic acid. *Yi chuan hered,* 42, 858–869.

Guardia, T., Rotelli, A. E., Juarez, A. O., & Pelzer, L. E. (2001). Anti- inflammatory properties of plant flavonoids. Effect of rutin, quercetin and hesperidin on adjuvant arthritis in rat. *Farmaco*, 56, 683–687.

Gullón, B., Lú-Chau, T. A., Moreira, M. T., Lema, J. M., & Eibes, G. (2017). Rutin: A review on extracrion, identification and purification methods, biological activities and approaches to enhance its bioavailability. *Trends food sci. technol,* 67, 220–235.

Gupta, C., Vikram, A., Tripathi, D. N., Ramarao, P., & Jena, G.B. (2010). Antioxidant and antimutagenic effect of quercetin against DEN induced hepatotoxicity in rat. *Phytother Res,* 24, 119–128.

Habas, R. R., Turker, M., & Ozdemir, F. A. (2019). *In vitro* Multiple Shoot Regeneration from Petunia hybrida. *Turkish journal of agriculture – food science and technology*, 7(10), 1554-1560.

Habib, R., Singh, S., & Bashir, I. (2018). Effect of cytokinins on *in vitro* propagation of Petunia hybrida hort. (vilm). *International journal of trend in scientific research and development*, 2(4), 1149-1152.

Hamood, A. K. & Majeed, B. H. (2017). Effect of benzyl adenine and salicylic acid on the growth and production of total alkaloids of Withania somnifera L ex vivo. *Iraqi journal of agricultural sciences*, 48 (1), 442.

Han, X., Zeng, H., Bartocci, P., Fantozzi, F., & Yan, Y. (2018) Phytohormones and Effects on Growth and Metabolites of Microalgae: A Review. *Article in fermentation*, 4- 25.

Hartmann, H. J., Kester, D. E., Geneve, R.L., & Davies, Jr. F. T. (1997). Plant Propagation: Principles and Practices. (Sixth edn.) Prentice - Hall Inc., New Jersey, USA.

Hartmann, H. T., Kester, D. E., Davies, F. T & Geneve, R. L. (2002). Plant Propagation Principles and Practices. 7th. Edition, Perntice Hall, Inc New Jersey. USA.

Hayat, S., Yadav, S., Ali, B., & Ahmad, A. (2010). Interactive effect of nitric oxide and brassino-steroids on photosynthesis and the antioxidant system of Lycopersiconesculentum. R*uss. j. plant physiol*. 57(2), 212-221.

Hertog, M.G., Feskens, E.J., Hollman, P.C., Katan, M.B., & Kromhout, D. (1993a). Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet,* 342, 1007–1011.

Heyn, C.C., & Joel, A. (1983). Reproductive relationships between annual species of Calendula. *Compositae,* 143(4), 311-329.

Hopkins, W. G., & Huner, N. P. A. (1995). Introduction to plant physiology. the University of Western Ontario. *John Wiley and Sons, Inc*, 449, 115-271.

Hou, D. X. (2003). Potential mechanism of cancer chemoprevention by anthocyanin. *Current advancements in molecular 369 medicines*, 3, 149–159.

Huang, Y., Fang, Y., Wu, J., Dziadyk, J.M., Zhu, X., Sui, M., Fan, W. (2004). Regulation of Vinca alkaloid-induced apoptosis by NF-kappaB/IkappaB pathway in human tumor cells. M*ol cancer ther,* 3, 271–277.

Huang, Z., Wang, Z., Shi, B., Wei, D., Chen, J., Wang, S., & Goa, B. (2015). Simultaneous Determination of Salicylic Acid, Acid Methyl Salicylate, and Methyl Jasmonate from Ulmus pumila Leaves by GC-MS. *J. of analytical chemistry*, 7.

Ibrahim, M. A., & Daraj, I. A. (2015). Micropropagation of dahlia plant Dahlia variabilis Wild (Desf.). Effect of explant and plant growth regulators on shoot regeneration and growth. A*dvances in agriculture & botanics international journal of the bioflux society*, 7 (1), 1-6.

Ioannou, M. (1989). Agricultural application of plant tissue culture: Rapidclonal propagation of

horticultural crops. Agric. Res. Ins. Ministryof Agriculture and Natural Resources. Nicosia, (Cyprus). *Miscellaneous Reports*, 37.

Irchhaiya, R., Kumar, A., Yadav, A., Gupta, N., Kumar, S., Gupta, N., & Gurjar, H. (2015). Metabolites in plants and its classification. *world j. of phar. and pharmace., sci*, 4(1), 287-305.

Jamal, M., Moniruzzaman, M., Shaheen, M.Z., Kamruzzaman Rashid, M., Mahmud, M., Khan, M.L., Pervin, N.A. & Sharif IH. H. (2014). Analysis of antimicrobial activity of Calendula arvensis against bacterial pathogens. *Int J phytother res,* 4:10–15.

Jaze', Liqa Ali (2013). Development of callus from the leaves of Nerium oleander and estimation of some of its qualitatively effective compounds inside and outside the body. *Journal of biotechnology research center*, 7(3), 12-17.

Johnson, M., & Lingakumar, K. (2011). Effect of crude brassinosteroid extract on growth and biochemical changes of *Gosssypium hirsutum* L. and *Vigna mungo* L. *Journal of stress physiology and biochemistry*, 7 (4): 324-334.

Kabera, J.N., Semana, E., Mussa, A.R., & He, X. (2014). Plant secondary metabolites: biosynthesis, classification, function and pharmacological properties. *J. of pharmacy and pharmacology,* 2, 377-392.

Karim, M. Z., Amin, M. N., Asaduzzaman, Islam, S., Hossin, F., & Alam, R. (2002). Rapid multiplication of Chrysanthemum morifolium through in vitro culture. *Pakistan journal of biological sciences*, 5(11), 1170-1172.

Karuppusamy, S. (2009). A.; Review on trends in production of secondary metabolites from higher plants. I*n vitro tissue, organ and cell culture journal of medicinal plants research*, 3, 1222–1239.

Kawaide, H. (2006). Biochemical and molecular analysis of gibberellin biosynthesis in fungi. *Bioscience, biotechnology and biochemistry*, 70, 583-590.

Kazemi, D., & Kaviani, B. (2017). Callus induction and organogenesis capacity from lamina explant of Petunia × hybrida F1 induced by BA and NAA. *Journal of ornamental plants*, 7(3), 157-162.

Khater, M. A. & Elashtokhy, M. M. A. (2015). Effect of growth regulatorson in vitro production of *Hyoscyamus aureus* L. and tropane alkaloids. I*nternational journal of chem tech research*, 8 (11), 113-119.

Kim, H., Kong H., Choi, B., Yang, Y., Kim, Y., Lim, M. J., Neckers, L., & Jung, Y. (2005). Metabolic and pharmacological properties of rutin, a dietary quercetin glycoside, for treatment of inflammatory bowel disease. *Pharm. res*, 22(9), 1499 –1509.

Klessig, D. F., Choi, H. W., & Dempsey, D. A. (2018). Systemic acquired resistance and salicylic acid: Past, present, and future. *Mol. plant microbe interact,* 31, 871–888.

Kumar, A., Sehgal, N., Kumar, P., Padi, S. S. V., & Naidu, P.S. (2008). Protective effect of quercetin against ICV colchicine-induced cognitive dysfunctions and oxidative damage in rats. *Phytother res,* 22, 1563–1569.

Kumar, S., & Singh, M. P. (2009). Plant Tissue Cultures. Published by S.B. Nangla for APH Publishing Corporation, New Delhi, India, P: 10.

Laemmel, E., Stucker, O., Pons, C., Duverger, J.P., Dedieu, F., & Leutenegger, E. (1998). Microcirculatory consequences of a venous striction in the rat. Effect of a coumarine-rutine association. *J mal vasc,* 23, 176–182.

Lee, H. I., Leon, J., & Raskin, I. (1995). Biosynthesis and metabolism of salicylic acid. *PNAS*, 92(10), 4076-4079.

Lefevere, H., Bauters, L, & Gheysen, G. (2020). Salicylic acid biosynthesis in plants. Front. *Plant sci,* 11: 338.

Li, W., & Fitzloff, J. F. (2001). High performance liquid chromatographic analysis of St. John's wort with photodiode array detection. *Journal of chromatography b: biomedical sciences and applications,* 765 (1), 99-105.

Lieselotte, D. B., H¨ofte, M., & De Vleesschauwer, D. (2014). Connect- ing growth and defense: the emerging roles of brassinosteroids and gibberellins in plant innate immunity. *Mol. Plant,* 7: 943– 959.

Lila, M. A. (2005). Valuable secondary products from in vitro culture. Chapter 24. Concept Secondary Products in vitro. CRC Press LLC.

Lin, J. K., Tsai, S. H., & Lin-Shiau, S. Y. (2001). Antiinflammatory and antitumor effects of flavonoids and flavanoids. *Drugs Fut,* 26, 145–150.

Lösel R., & Wehling M. (2003). Non-genomic actions of steroid hormones. *Nat. rev. mol. cell. bio*, 4, 46 –54.

Lovegrove, A., Barratt, D. H., Beale, M. H., & Hooley, R. (1998). Gibberellin-photoaffinity labelling of two polypeptides in plant plasma membranes. *The plant journal: for cell and molecular biology,* 15 (3), 311–320.

Mahesh, S. (2008). Plant Molecular Biotechnology. New Age International (P) Ltd., Publishers 1st. Edit. Pp. 99-101.

Malikova, J., Swaczynova, J., Kolar, Z., & Strnad, M. (2008). Anticancer and antiproliferative activity of natural brassinosteroids. *Phytochem*, 69: 418-426.

Mandal, S. M., Chakraborty, D., & Dey, S. (2010). Phenolic acids act as signaling molecules in plant-microbe symbioses. *Plant signal. behav,* 5, 359 –368.

Marcinek, B., Parzymies, M., Poniewozik, M., Kozak, D., & Durlak, W. (2019). The influence of growth regulators on Dahlia propagation in tissue culture and acclimatization of plants in ex vitro conditions. A*cta scientiarum polonorum hortorum cultus*, 18 (5), 49-61.

Margl, L., Tei, A., Gyurjan, I., & Wink, M. (2002). GLC – MS analysis of thiophene derivatives in plant and In vitro culture of Tagetes patula (Asteraceae). *Z. naturforsch*, 57, 63-71.

Mauludin, R., Müller, R. H., & Keck, C. M. (2009). Development of an oral rutin nanocrystal formulation. *Int. j. pharm,* 370(1-2), 202-209.

Mehri, S., Ilkaee, M. N., & Saeedzadeh, F. (2018). A Simple and Rapid Method for Micropropagation of Petunia × hybrida F1 'Opera Supreme Pink Morn'. *Journal of ornamental plants*, 8 (4), 255-263.

Métraux, J. P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W., & Inverardi, B. (1990). Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. *Science*, 250, 1004–1006.

Metwally, A., Finkemeier, I., Georgi, M., & Dietz, K. J. (2003). Salicylic acid alleviates the cadmium toxicity in barley seedlings. *Plant physiol,* 132: 272–281.

Mitchell, J. W., Mandhava, N. B., Worley, J. F., Plimmer, J. R., & Smith, M. V. (1970). Brassins − a new family of plant hormones from rape pollen. *Nature,* 255: 1065-1066.

Muhammad, A. M. S., & Mubashir, S. O. (1990). The main concepts in the cultivation of cells, tissues and organs of plants, Ministry of Higher Education and Scientific Research - University of Mosul. Iraq.

Muhammad, J. H., (2003). The Basics of Plant Physiology. *Al-Dar Al-Jadeeda*, 352.

Muley, B. P., Khadabadi, S. S., & Banarase, N. B. (2009). Phytochemical Constituents and Pharmacological Officinalis Linn (Asteraceae): *A review. j. pharm. Res*, 8(5), 455-465.

Murashige, T., & Skoog, F. (1962). A resived medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. plant*, 15:473-497.

Naeem, M., Idrees, Alam, M., Aftab, M. M., Khan, T., & Moinuddin, M.M. (2012). Brassino-steroid-mediated enrichment in yield attributes, active constituents and essential oil production in Menthaarvensis L. *Russ. agric. sci*, 38: 106-113.

Nakamura, H., Furukawa, F., Nishikawa, A., Miyauchi, M., Son, H. Y., & Imazawa, T. (2001). Oral toxicity of a tocotrienol preparation in rats. *Food chem toxicol,* 39: 799 –805.

Nalawade, S., & Tsay, H. S. (2004). *In vitro* propagation of some important Chinese medicinal plants and their sustainable usage. In vitro cellular and development biology – plant, 40(2), 143- 154.

Nalini, R., & Nagar, G. B. (2012). Micropropagation of Chrysanthemum (Chrysanthemum morifolium) using shoot tip as explant. *International `journal of food agriculture and veterinary sciences*, 2(2), 62-66.

Namdeo, A. G. (2007). Plant cell elicitation for production of secondary metabolites: *A review. pharamacognosy rev*, 1(2-3), 69-79.

Nemhauser, J. L., Mockler, T. C., Chory, J., & Dangl, J. (2004). Interdependency of brassinosteroid and auxin signaling in Arabidopsis. *PLoS Biol*, 2(9), 258.

Oh, M. H., & Clause, S.D. (1998). Brassinolide affects the rate of cell division in isolated leaf protoplasts of Petunia hybrida. *Plant cell rep*, 17, 921-924.

Paolini, J., Barboni, T., Desjobert, J. M., Djabou, N., Muselli, A., & Costa, J. (2010). Chemical composition, intraspecies variation and seasonal variation in essential oils of Calendula arvensis L. *Biochem syst ecol,* 38, 865–874.

Park, S. U., Uddin, M. R., Xu, H., Kim, Y. K., & Lee, S. Y. (2008). Biotechnological applications for rosmarinic acid production in plant. *African j. of biotechnology*, 7(25), 4959 - 4965.

Passalacqua, N., Guarrera, P., & De Fine, G. (2007). Contribution to the knowledge of the folk plant medicine in Calabria region (Southern Italy). *Fitoterapia*, 78, 52–68.

Prior, R. L. (2004). Absorption and metabolism of anthocyanins: Potential health effects. In M. Meskin, W. R. Bidlack, A. J. 1-19 ref 59.

Qassab Bashi, B. Z. A., & Al-Mamari, A. M. S. S. (2010). The effect of kinetin on the emergence and multiplication of the tips of the branches and nodes of the carnation Dianthus caryophyllus ex vivo. *Journal of rafidain agriculture*, 38(1).

Qassab Bashi, B. Z., & Yahya, K. M. (2017). Duplication of branch tips and nodes of *Calendula officinalis* L. and the emergence and differentiation of callus ex vivo. *Journal of mesopotamia agriculture*, 45(1), 103- 112.

Qassab Bashi, B. Z. A., & Al-Mamari, A. M. S. S. (2011). Effect of BA and TDZ on the doubling of the tips of the branches and nodes of carnation Dianthus caryophyllus ex vivo. T*ikrit university journal of agricultural sciences,* 11(1), 126-135.

Radovanovic, B., Mladenovic, J., Radovanovic, A., Pavlovic, R., & Nikolic, V. (2015). Phenolik Composition, Antioxidant, Antimicrobial and Cytotoxic Activites of Allium Porrum L. (Serbia) Extracts, *J. of food and nutrition research,* 3(9), 564 – 569.

Ramawat, K. G. (2004). Plant Biotechnology. S. Chand and company LTD. Ram Nagar, New Delhi, India.

Raskin, I. (1992). Role of salicylic acid in plants. A*nnu. rev. plant physiol. plant mol. Biol,* 43, 439–463.

Raskin, I. (1992). Salicylate, A New Plant Hormone. *Plant physiol,* 99, 799–803.

Raskin, I., Ehmann, A., Melander, W. R. & Meeuse, B. J. D. (1987). Salicylic acid: A natural inducer of heat production in Arum lilies. *Science*, 237, 1601–1602.

Reddy, P. S. & Jamil, K. (2001). P Madhusudhan. *Pharmaceutical Biol*, 39: 236 –8.

Reis, M. S., & Mariot, A. (1999). Diversidade natural e aspectos agronômicos de plan-tas medicinais. In: Simões CMO, editor. Farmacognosia: da planta ao medicamento. P*orto alegre: florianópolis, universidade*, 39–41.

Rejšková, A., Brom, J., Pokorný, J., & Korečko, J. (2010). Distr. Func. Ecol. Plants. J. Flora (Jena), 205(4): 282-289.

Rivas-San Vicente, M., & Plasencia, J. (2011). Salicylic acid beyond defence: Its role in plant growth and development. *J. exp. Bot,* 62, 3321-3338.

Rokosa, M. T., & Kulpa, D. (2020). Micropropagation of Stevia rebaudiana plants. *Ciencia Rural, Santa Maria*, 50 (1), 1-9.

Ruiz de Clavijo, E. (2005). The reproductive Strategies of the heterocarpic annual calendula arvensis (*Asteraceae*). *Acta. Oecol*, 28(2), 119-126.

Salman, M. A. (1988). Fundamentals of plant cell and tissue culture. *The Ministry of Higher Education and Scientific Research / University of Baghdad. Iraq*.

Santoro, M. V., Nievas, F., Zygadlo, J., Giordano, W., & Banchio, E. (2013). Effects of growth regulators on biomass and the production of secondary metabolites in peppermint (*Menthapiperita*) Micropropagated in Vitro. *Am. j. plant sci*. 4, 49-55.

Santos-Buelga, C., & Williamson, G. (2003). Methods in Polyphenol Analysis, The Royal Society of Chemistry, Cambridge.

Sasse, J. M. (2003). Physiological Actions of Brassinosteroids: An Update. *J. plant growth regul*, 22: 276-288.

Sasson, A. (1991). Production of useful biochemicals by higher-plant cellcultures: Biotechnological and economic aspects. *CIHEAM-Options Mediterraneennes (UNESCO)*,59-74.

Seyfferth, C., Tsuda, K. (2014). Salicylic acid signal transduction: The initiation of biosynthesis, perception and transcriptional repro-gramming. *Front. plant sci,* 5: 697.

Shi, Y. L., Sheng, Y. Y., Cai, Z. Y., Yang, R., Li, Q. S., Li, X. M., Li, D., Guo, X. Y., Lu, J. L., & Ye, J. H. (2019). Involvement of salicylic acid in anthracnose infection in tea plants revealed by transcriptome profiling. *Int. j. mol. sci,* 20, 2439.

Shohji, T.; Winz, R.; Iwase, T.; Nakajima, K.; Yamada, Y. and Hashimoto, T. (2002). Expression patterns of two tobacco isoflavone reductase– likegenes and their possible roles in secondary metabolism in tobacco. Plant Mol Biol 50: 427-440.

Smith, R. H. (2000). Plant Tissue Culture Techniques and Experiments 2nd edition. department of soil and crop sciences texas A&M University collage station texas. *International standard book number*.

Sohair, K. H., El-Saeid, H. M., & Shalaby, M. (2006). The role of kinetin in flower abscission and yield of lentil plant. *J. of appl. sci. res*, 2(9), 587- 591.

Stirk, W. A., Tarkowská, D., Turečová, V., Strnad. M., & Staden, J.V. (2014) Abscisic acid, gibberellins and brassinosteroids in Kelpak, a commercial seaweed extract made from Ecklonia maxima. *Journal of applied phycolog* y, 26(1), 561- 567.

Strobel, N. E., & Kuc, J. A. (1995). Chemical and biological inducers of systemic resistance to pathogens protect cucumber and tobacco plants from damage caused by paraquat and cupric chloride. *Phytopathology*, 85: 1306–1310.

Swamy, K. N., & Rao, S. S. R. (2008). Influence of 28-Homobrassinolide on growth, photosynthesis metabolite and essential oil content of geranium (Pelargonium graveolens (L.) Herit.). *Amer. j. plant physiol*, 3: 173-179.

Taiz, L., & Zeiger, E. (2003). Plant Physiology. 3rd edn. Sunderland Sinauer Associates. Pp. 750-751.

Tawfik, A. A., & Taha, M. A. A. (2019). Enhanced indirect organogenesis and efficient shoot multiplication of Petunia hybrida. *Egyptian Journal of Horticulture*, 46 (1),13-28.

Thakur, M., & Sohal, B.S. (2013). Role of elicitors in inducing resistance in plants against pathogen infection: A review. *ISRN biochem*, 1-10.

Thirukkumaran, G., Ntui, V. O., Khan, R. S., & Mii, M. (2009). Thidiazuron: an efficient plant growth regulator for enhancing Agrobacterium- mediated transformation in Petunia hybrida. *Plant cell tissue and organ culture*, 99: 109–115.

Tiwari, S. (2008). Plants: a rich source of herbal medicine. *J nat prod*, 1:27–35.

Tosun, G., Yayli, B., Arslan, T., Yasar, A., Karaoglu, S. A., & Yayli, N. (2012). Comparative essential oil analysis of Calendula arvensis L. Extracted by hydrodistillation and microwave distillation and antimicrobial activities. *Asian j chem,* 24:1955.

Towers, G.H., Lopez, A., & Hudson, J.B. (2001). Antiviral and antimicrobial activitiec of medicinal plants. *J. Ethnopharmacol,* 77:189 – 96.

Vakili, A. N., Bagheri, H., & Azadi, P. (2019). Direct shoot regeneration of three Petunia cultivars. *Advances in Horticultural Science*, 33 (3), 375- 379.

Van Butselaar, T., & Van den Ackerveken, G. (2020). Salicylic acid steers the growth-immunity tradeoff. *Trends plant sci,* 25: 566–576.

Van Hung, P. (2016). Phenolic compounds of cereals and their antioxidant capacity. *Crit. rev. food sci. nutr,* 56: 25–35.

Vanisreel, M., Lee, C., Nalawadel, S., lin, C., & Tsay, H. (2004). Studies on the production of same important secondary metabolites from medicinal plants by plant tissue cultures. *Plant biotech. bull acad. Sin*, 45:1-22.

Vlot, A.C., Dempsey, D.A., & Klessig, D.F. (2009). Salicylic acid, a multifaceted hormone to combat disease. *Annu. rev. phytopathol*, 47: 177–206.

Vriet, C., Russinova, E., & Reuzeau, C. (2012). Boosting crop yields with plant steroids. *Plant cell* 24:842-857.

Wallis, C. M., & Galarneau, E. R. A. (2020). Phenolic Compound induction in plant-microbe and plant-insect interactions: A meta-analysis. *Front. plant sci*, 11: 2034.

Wasfi, E.H. (1995) Growth and flowering plants and their use in acanthosis nigricans. *Cairo- Egypt*.

Weissmann, G. (1991). Aspirin. *Sci. Am*, 264: 84 –91.

Wiesman, Z., Riov, J., & Epstein, E. (1989). Characterization and rooting ability of indol-3-butyric acid conjugates formed during rooting of mung bean cuttings. *Plant physiol*, 91: 1080-1084.

Xia, X. J., Huang, Y. Y., Wang, L., Huang, L. F., Yu, Y. L., Zhou, Y. H, & Yu, J. Q. (2006). Pesticides induced depression of photosynthesis was alleviated by 24-epibrassinolide pretreatment in Cucumissativus L. *Pest. bioch. physiol*, 86: 42-48.

Yadav, R. (2012). Phytochemical Screening of Spilanthes acmella plant parts. *International journal of pharmaceutical erudition,* 1: 44-72.

Yang, J., Guo. J., & Yuan, J. (2008). In vitro antioxidant properties of rutin. *LWT- Food Sci. Technol,* 41(6), 1060–1066.

Yang, T., Kong, B., Gu, J., W., Kuang, Y. Q., Cheng, L., Yang, W. T., Xia, X., & Shu, H. F. (2014). *Cell. mol. neurobiol,* 34: 797–804.

Yao, Y., Han, D. D., Zhang, T., & Yang, Z. (2010). Quercetin improves cognitive deficits in rats with chronic cerebral ischemia and inhibits voltage-dependent sodium channels in hippo-campal CA1 pyramidal neurons. *Phytother res,* 24:136–140.

Zhao, J., Hu, Q., Guo, & Zhn, W. H. (2001a). Enhanced Catharanthine Production in *Catharathus roseus* cell cultures by combined elicitortreatment in shake flasks and bioreactors. *Encyme. Microb. Technol*, 28: 673-681.

Zhao, J., Zhu, W. H., Hu, Q., & Guo, Y. Q. (2001b). Compact callus cluster suspension cultures of *Catharanthus roseus* with enhanced indole alkaloid biosynthesis. *In vitro cell dev. biol-plant*, 37: 68-72.

Zurek, D. M., Rayle, D. L., McMorris, T. C., & Clouse, S. D. (1994). Investigation of gene expression, growth kinetics, and wall extensibility during brassinosteroid regulated stem elongation. *Plant physiol*, 104: 503-513.