T.C.

ÇANAKKALE ONSEKIZ MART UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

OPTIMIZATION OF PLANT TISSUE CULTURE SYSTEM OF *Galanthus trojanus*

MASTER OF SCIENCE THESIS

MEHMET ÖZKIR

Thesis supervisor:

Assoc. Prof. Özge KARAKAŞ METİN

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ETHICAL STATEMENT

Çanakkale Onsekiz Mart Üniversitesi Lisansüstü Eğitim Enstitüsü Tez Yazım Kuralları'na uygun olarak hazırladığım bu tez çalışmasında; tez içinde sunduğum verileri, bilgileri ve dokümanları akademik ve etik kurallar çerçevesinde elde ettiğimi, tüm bilgi, belge, değerlendirme ve sonuçları bilimsel etik ve ahlak kurallarına uygun olarak sunduğumu, tez çalışmasında yararlandığım eserlerin tümüne uygun atıfta bulunarak kaynak gösterdiğimi, kullanılan verilerde herhangi bir değişiklik yapmadığımı, bu tezde sunduğum çalışmanın özgün olduğunu, bildirir, aksi bir durumda aleyhime doğabilecek tüm hak kayıplarını kabullendiğimi taahhüt ve beyan ederim.

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Mehmet ÖZKIR
25/08/2022

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Mehmet ÖZKIR

Canakkale, August 2022

ÖZET

Galanthus trojanus İÇİN BİTKİ DOKU KÜLTÜRÜ SİSTEMİNİN OPTİMİZASYONU

Mehmet ÖZKIR
Çanakkale Onsekiz Mart Üniversitesi
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Moleküler Biyoloji ve Genetik Anabilim Yüksek Lisans Yeterlik Tezi
Danışman: Doç. Dr. Özge KARAKAŞ METİN
25/08/2022, 63

Truva Kardeleni olarak adlandırılan *Galanthus trojanus*, Amaryllidaceae familyasından gösterişli ve zarif çiçekleri olan soğanlı bir bitki türüdür. Bu bitki Çanakkale ilinin Bayramiç ve Çan olmak üzere iki farklı bölgesinde yetişmektedir. *Galanthus trojanus* endemik bir bitkidir ve alkaloid bileşikler olarak adlandırılan çok değerli sekonder metabolitler üretir. Stylopin, protopin, nikotinik asit, tiramin ve likorin bu bileşiklerden birkaçıdır. Bu alkaloid bileşikler önemli biyolojik özelliklere sahiptirler ve farmakoloji endüstrisinde birçok farklı hastalığa karşı ilaç elde etmek için kullanılırlar. Öte yandan bu bitki türü, süs değeri taşıması ve yöre halkı tarafından geleneksel olarak kullanılması nedeniyle ekonomik olarak da önemlidir.

Galanthus trojanus'un gösterişli ve zarif çiçeklere sahip olması sebebiyle doğadan bilinçsiz bir şekilde çıkarılıp toplanması sayısının hızlı bir şekilde azalmasına neden olmuştur. Bu nedenle 2011 yılında *Galanthus trojanus*, IUCN tarafından kritik olarak tehlike altındaki türlerin B1 listesine dahil edildi. *Galanthus trojanus*'un yok olmasının önüne geçmek için uygun çoğaltma yöntemlerinin araştırılması önem arz etmektedir.

Bitki doku kültürü sistemi, tehdit ve tehlike altındaki canlı bitki genotiplerini korumak ve büyük miktarlarda çoğalmak için kullanılabilecek bir tekniktir. *Galanthus trojanus* nesli tükenmekte olan bir bitki türü olduğundan, bitki doku kültürü protokolü oluşturulmalı ve optimize edilmelidir. Bugüne kadar *Galanthus trojanus'*un bitki doku kültürü sistemini

kurmak amacıyla bir çalışma gerçekleştirilmemiştir. Bu nedenle hazırlanan tez kapsamında öncelikle *Galanthus trojanus* yüzey sterilizasyon protokolü geliştirilmiş, daha sonra da soğan eksplantları kullanılarak G*alanthus trojanus'*un bitki doku kültürü protokolünün optimizasyonuna yönelik çalışmalar gerçekleştirilmiştir. Bu eksplantlar farklı konsantrasyonlarda 6-Benzylaminopurine (BAP) ve Naftalin asetik asit (NAA) bitki büyüme düzenleyicilerini içeren Murashige Skoog (MS) besiortamında incelenmiştir. Bu sayede, farklı dozlardaki bitki büyüme düzenleyicilerin *Galanthus trojanus*'un rejenerasyonu üzerindeki etkisi araştırılmıştır.

Tez kapsamında gerçekleştirilen çalışmalar sonucunda 0,5 mg/l BAP ve 0,1 mg/l NAA içeren Murashige ve Skoog (MS) besi ortamı gövde oluşumu için en uygun besiyeri olarak belirlenmiştir.

Anahtar Kelimeler: Amaryllidaceae ailesi, Endemik bitki, *Galanthus trojanus*, Bitki doku kültürü, Sekonder metabolitler, Tehlike altındaki bitki

ABSTRACT

OPTIMIZATION OF PLANT TISSUE CULTURE SYSTEM OF *Galanthus trojanus*

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Master of Science Thesis in Molecular Biology and Genetic

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25/08/2022, 63

Galanthus trojanus, called Trojan Snowdrop, is a bulbous plant species with showy and elegant flowers in the Amaryllidaceae family. This plant grows in two different regions of Çanakkale province: Bayramiç and Çan. *Galanthus trojanus*, which produces very precious secondary metabolites, especially alkaloid compounds, is an endemic plant. Stylopine, protopine, nicotinic acid, tyramine, and lycorine are a few of these compounds. They have important biological properties and are used in the pharmacological industry to acquire drugs against many diseases. On the other hand, these plant species are economically important because they have an ornamental value and are traditionally used by local people.

Galanthus trojanus has beautiful views, so they are gathered and removed unconsciously from nature leading to a rapid decrease in the quantity of *Galanthus trojanus*. So, in 2011, *Galanthus trojanus* was on the B1 list of critically endangered species by the IUCN. To prevent the extinction of the *Galanthus trojanus* species, investigation of appropriate propagation methods are essential.

A plant tissue culture system is a technique that can be used to conserve living plant genotypes that are threatened and endangered and produce large amounts of them. Because the *Galanthus trojanus* is an endangered plant species, plant tissue culture protocol for *Galanthus trojanus* should be established and optimized. To date, no study has been conducted on the plant tissue culture system of *Galanthus trojanus*. So, in this study, an

efficient surface sterilization method was developed after that optimized the plant tissue culture protocol of *Galanthus trojanus* by using bulb scale explants. These explants are grown in MS medium with varying doses of BAP (6-Benzyl Amino Purine) and NAA (Naphthalene acetic acid) plant growth regulators. In this manner, the impact of plant growth regulators (PGR) at various doses on *Galanthus trojanus* regeneration in tissue culture was investigated.

Murashige and Skoog (MS) medium supplemented with the combination of 0.5 mg/l BAP and 0.1mg/l NAA was the most suitable treatment medium for shoot formation.

Keywords: Amaryllidaceae family, Endemic plant, *Galanthus trojanus*, Plant tissue culture, Secondary metabolites, Endangered plant

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SYMBOLS AND ABBREVIATIONS

ABA	Abscisic acid
BA	Benzylaminopurine
cm^3	Cubic centimeter
^oC	Degrees Celsius
C	Concentration
DNA	Deoxyribonucleic Acid
dH2O, dWater	Distilled water
2,4-D	2,4-Dichlorophenoxyacetic acid
et al.	Friends
g/l	Gram/liter
HCl	Hydrochloric acid
$HgCl_2$	Mercuric chloride
IAA	Indole-3-acetic acid
KN	Kinetin
LS	Linsmaier and Skoog
LED	Light-emitting diodes
µl	Microliter
µM	Micromolar
mg/L	Milligram/liter
ml	Milliliter
Min	Minute
Mol	Molar
MS	Murashige and Skoog
N	Normal
NAA	Naphthalene acetic acid
NaOH	Sodium hydroxide
NaOCl	Sodium hypochlorite
PEG	Polyethylene glycol
PGR	Plant Growth Regulator
pH	Potential of hydrogen

Ppm	parts per million
PVP	Polyvinylpyrrolidone
PPM	Plant preservative mixture
%	Percent
RNA	Ribonucleic acid
Sec	Second
V	Volume

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explants in a 90-day period

CHAPTER 1

INTRODUCTION

Flower bulbs, also known as ornamental geophytes, are a complex set of morphological, physiological, and biological growth forms in the plant kingdom (Raunkiaer, 1934). They make an essential contribution to the international ornamental plant trade. They are utilized in indoor and outdoor floral decorations such as beautiful flowers and potted plants or unspotted plants in gardens, landscaping, and soil conservation activities in various locations (Kızıl et al., 2012).

Most flower bulbs can significantly contribute to the pharmaceutical and ornamental plant sectors. Due to the absence of systematically collected information about these plants, they could not be appropriately appraised. People in the area eat the leaves, bulbs, and blossoms as salads or vegetables and use them to make pharmaceutically significant products (Kızıl et al., 2012). For example, some species of *Amaryllidaceae* and *Hyacinthaceae* families have used disinfectants and anti-inflammatory agents in South Africa (Louw et al., 2002).

Conventional flower bulbs are typically grown in regions of the world with temperate climates (Buschman, 2004). Furthermore, as global demand for all bulbous flower plants grows, it is clear that innovative manufacturing and sales strategies are needed. This is correct not only for the significant genera but also for the wide variety of minor taxa.

Even though there are over 800 different species of flower bulbs, seven are dominant in the global sector: Hyacinthus, *Lilium, Narcissus, Tulipa, Gladiolus, Crocus,* and *Iris.* Also, *Amaryllidaceae, Orchidaceae, Hyacinthaceae, Iridaceae, Araceae, Liliaceae,* and *Ranunculaceae* plant families dominate the flower sector in Turkey.

The most important plant species for trade are *Cyclamen, Eranthis, Anemone, Leucojum,* and *Galanthus.* Because of a variety of biotic and abiotic stress factors, the wild ecosystem of some of these plant species has declined. As a result, the states have controlled their use by prohibiting the export of these plants and excavating them for their bulbs or roots (Zencirkiran and Gürbüz, 2009).

Flower bulbs or ornamental geophytes used in commercial floriculture and landscaping necessitate steady and fast reproduction. Even though most geophyte species reproduce vegetatively, their reproduction rate is frequently poor due to a limited number of axillary meristems. As a result, the introduction of newly bred cultivars or pathogen-free propagation material necessitates a more extended period for the development of commercial stock.

Because their habitats are damaged due to climate change and export of them for ornamental and pharmaceutically property, and thus, many bulbous species are extinction. To prevent the extinction of these many bulbous plant species and increase the number of individuals in species to obtain many more pharmacological compounds they produce, we should establish an in vitro regeneration system protocol.

1.1. Amaryllidaceae Family

Around 1000 species and 85 bulbous genera exist in the Amaryllidaceae family, found in tropical, generally warm areas. These bulbous species are reputed for their typical applications and decorative appeal. Moreover, they produce very precious secondary metabolites called Amaryllidaceae alkaloid compounds (Babashpour-Asl et al., 2016). These alkaloid compounds have numerous biological activities, pain relief, antibacterial, and antimalarial, and also exhibit anticancer properties and effects on the central nervous system (von Linné, 2007).

1.1.1. The Genus *Galanthus*

Galanthus (known as Snowdrop) is a member of the Amaryllidaceae family throughout Europe, Asia Minor, and the Caucasus region (Davis, 1999). Some species are found worldwide, while others are only in a few places. *Galanthus nivalis*, for instance, is abundant worldwide, whereas *Galanthus trojanus* is an uncommon wild species located in the Çanakkale province of Turkey (Davis and Ozhatay, 2001). Turkey is one of the countries with the most species geographically (Ünver, 2007).

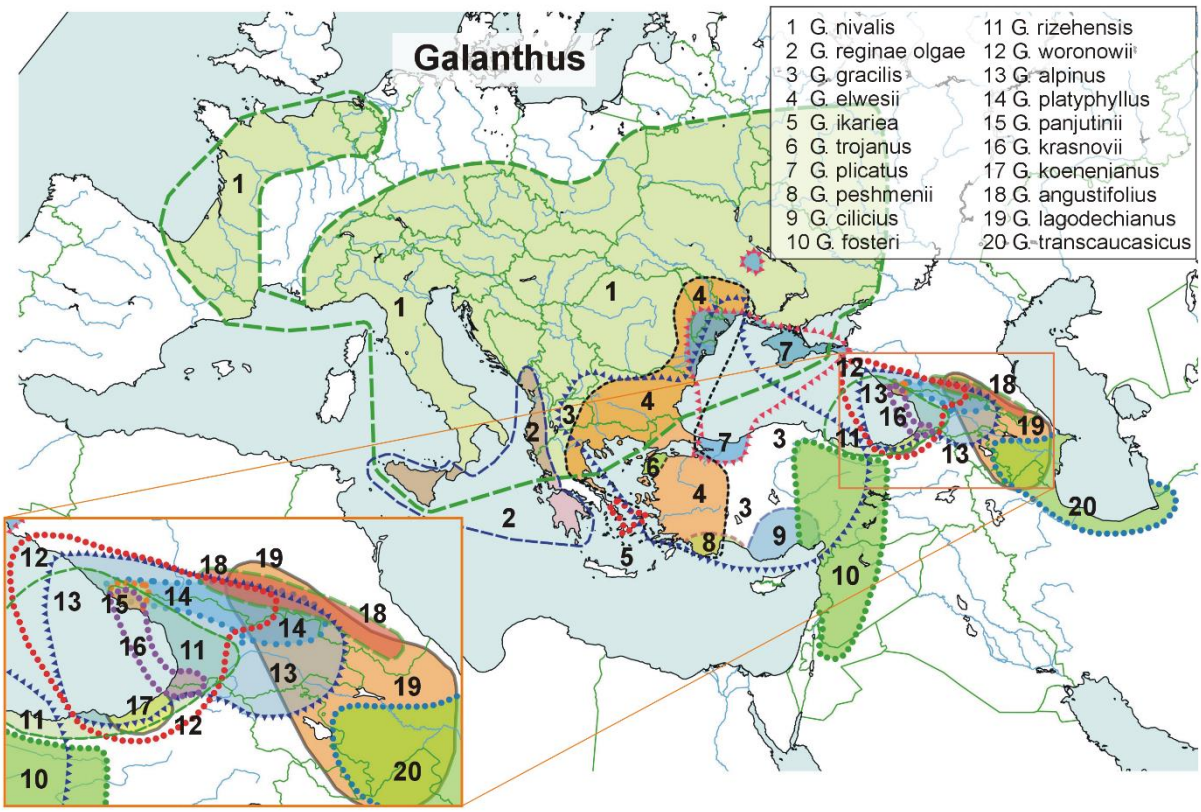


Figure 1. Dispersion of *Galanthus* species throughout Europe and Western Asia (Davis, 1999).

The genus *Galanthus* is economically significant due to its ornamental value and uses as a landscaping plant (Semerdjieva et al., 2019). Also, *the Galanthus* species produces beneficial secondary metabolites, which have biological functions used in the medical industry to produce medicaments. Many diseases can or will be cured thanks to these metabolites; thus, they are very precious. Phenolics, flavonoids, and likorin are a few of them (Semerdjieva et al., 2019). Many different metabolites extracted from *Galanthus* species have been found to exhibit antibacterial, anticancer, antifungal, antiviral, antioxidant, and anti-inflammatory effects throughout the previous three decades (Elgorashi et al., 2003; Orhan and Şener, 2003; Ločárek et al., 2015; Resetár et al., 2017). Galanthamine and lycorine are Galanthus's main pharmacologically active constituents, particularly in the bulbs (Ayaz et al., 2019). Galanthamine, for example, is an acetylcholinesterase inhibitor that is selective, reversible, and competitive (Thomsen et al., 1998) and used to treat Alzheimer's and other neurological diseases (Heinrich and Teoh, 2004).

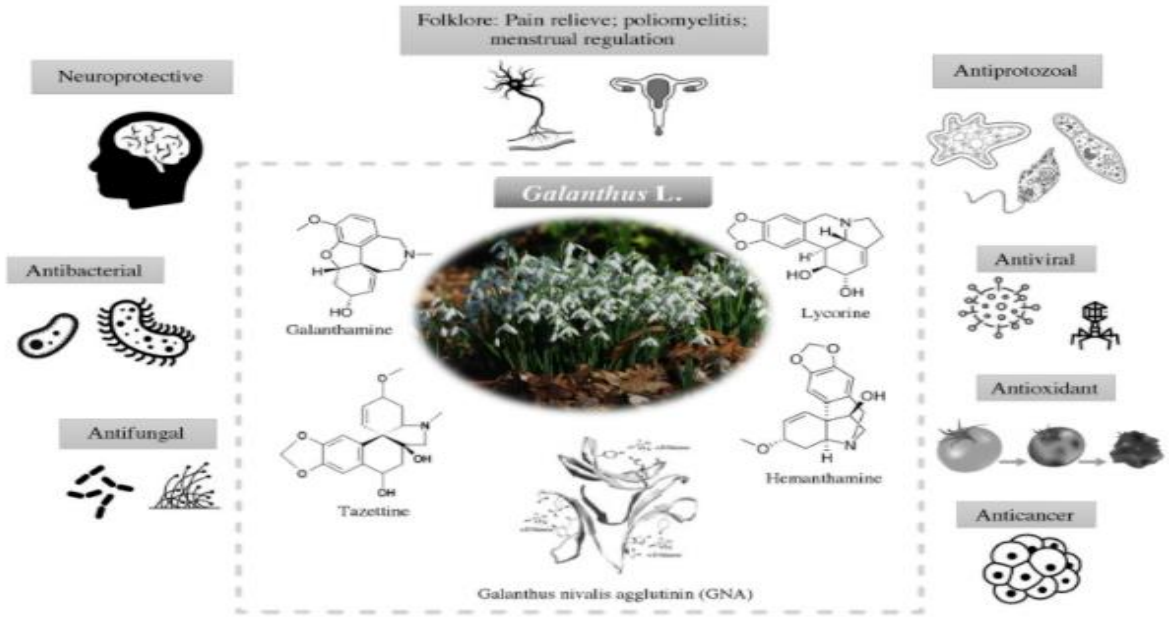


Figure 2. Biological activities of alkaloid compounds produce by *Galanthus* species (Kong et al., 2021).

Galanthus species are herbaceous perennials that develop from bulbs. Their blooms have six tepals, with the external three being more prominent and convex than the internal series. A green or greenish-yellow bridge-shaped mark at the apex of each tepal marks the internal bloom parts. The ovary comprises three cells and develops into a three-celled capsule (Davis, 1999).

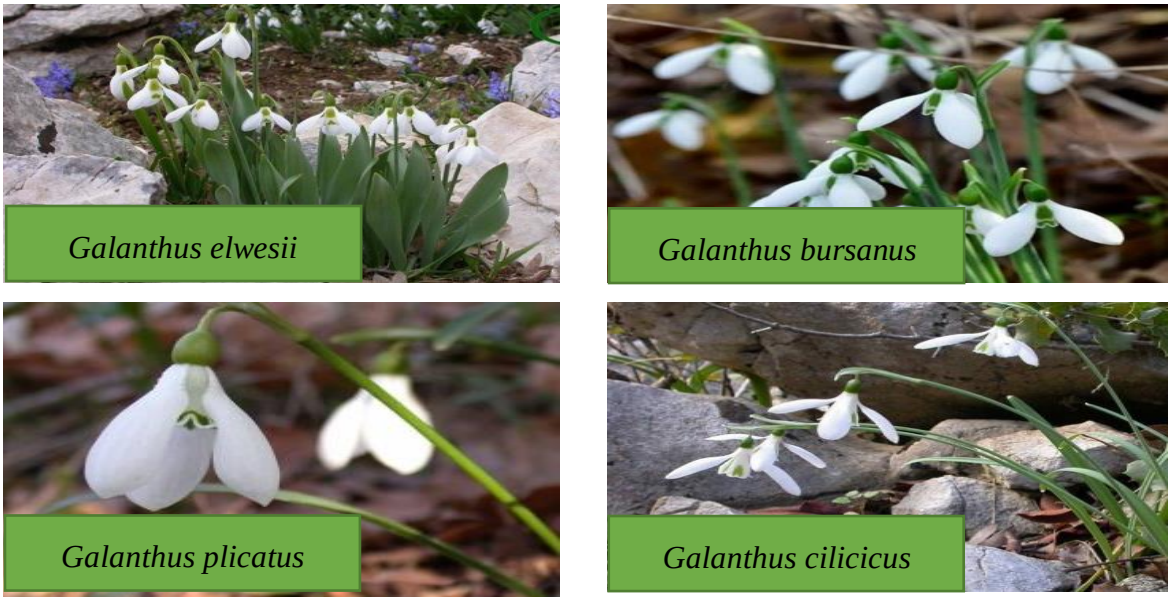


Figure 3. Various *Galanthus* species (Plants of Turkey, 2018).

Because of a lack of well-defined physical traits and a high level of diversity, *Galanthus* species are challenging to recognize and characterize (Davis and Barnet, 1997). There are a few different approaches have been applied to distinguish its species from each other, which are gone), anatomical (Davis and Barnet, 1997), and DNA-based molecular (Zonneveld et al., 2003).

Galanthus is estimated to include 19 species, six cultivars, and two natural interspecies hybrids. (World Checklist of Selected Plant Families, 2011). There are more than eleven alkaloid compounds found in the *Galanthus* genus. Even though the genus has only been studied to a limited extent, the phytochemical investigation discovered various remarkable metabolites. Also, some of these metabolites have unknown bioactivities yet (Berkov et al., 2012).

In Turkey, 17 different *Galanthus* species are identified. Among 17 different *Galanthus* species, G. *plicatus* ssp. *byzantinus, G. cilicicus, G. elwesii, G. koenenianus, G. trojanus, G. xvalentinei, G. bursanus* are endemic. Most species are found in woodlands, typically in mountainous areas (above 1000 m) and forests. They like cold, often shady conditions with enough water during the cultivation period. *Galanthus's* growth altitude ranges from sea level to around 2700 m (Taşçi, 2004).

Table 1

Galanthus Species that grow in Turkey

Galanthus trojanus	***Galanthus. rizehensis***
Galanthus fosteri	***Galanthus alpinus***
Galanthus nivalis	***Galanthus woronowii***
Galanthus xvalentinei	***Galanthus plicatus ssp plicatus***
Galanthus gracilis	***Galanthus elwesii***
Galanthus cilicicus	***Galanthus peshmenii***
Galanthus peshmenii	***Galanthus plicatus* ssp *byzantinus***
Galanthus krasnowii	***Galanthus bursanus***
Galanthus koenenianus	

Galanthus trojanus

Galanthus trojanus is an endemic plant that occurs in northwestern Turkey. The name relates to the expanding region in the eastern part of the Troas landscape, which surrounded the ancient city of Troy. Currently, the region is the province of Çanakkale. Davis and Ozhatay first identify it. Also, *Galanthus trojanus* is called a Trojan Snowdrop by local people.

Galanthus trojanus grows at elevations ranging from 300 to 500 meters. It has bright green leaves and applanate vernation with a single mark at the apex of each inner petal. They often produce two scapes per bulb. *Galanthus trojanus* flowers in March, whereas in cultivation, flowering occurs from January to February (Davis and Ozhatay, 2001).

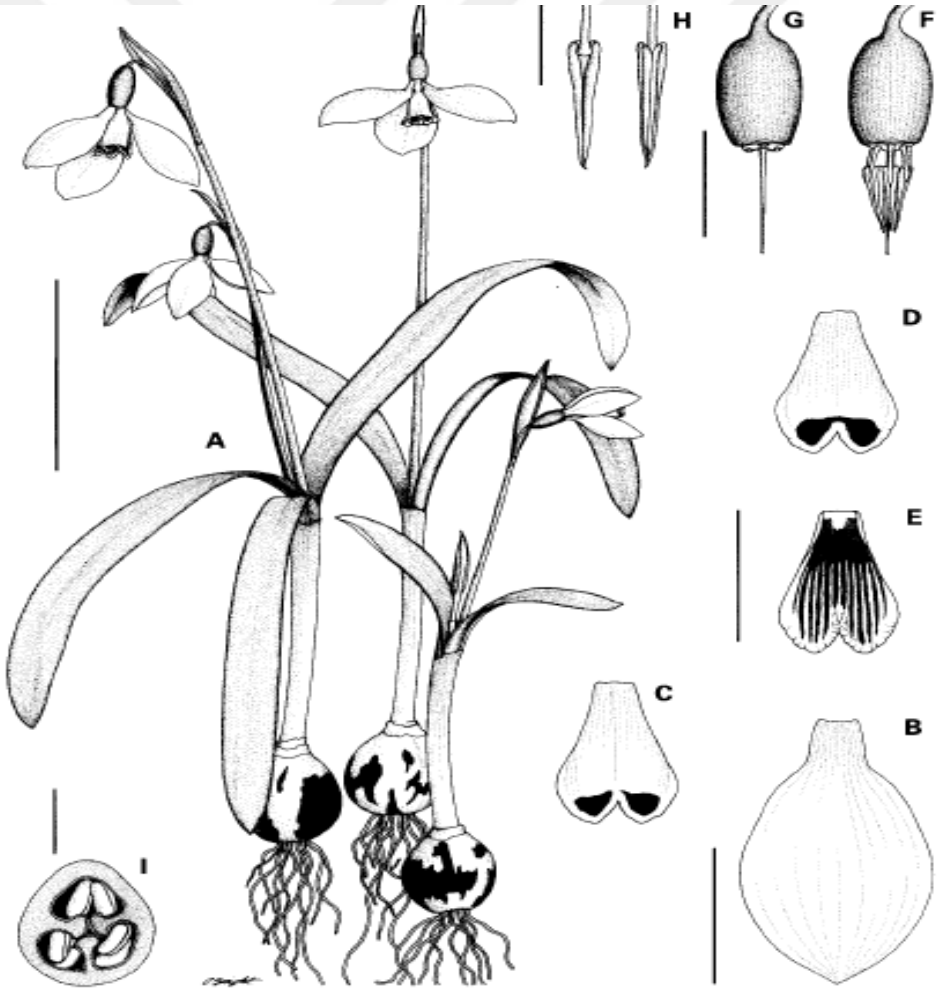


Figure 4. *Galanthus trojanus* sp. nov. A habit. B, outer perianth segment. C and D, inner perianth segment (outer surface). E, inner perianth segment (inner surface). F, ovary, stamens, and style. G, ovary, with stamens removed. H, anthers, abaxial and adaxial views (left to right). I, transverse section through the ovary (Davis and Ozhatay, 2001).

Galanthus trojanus is a fantastic species since the latest phylogenetic analysis investigations have revealed that this snowdrop diverged from the other members of the genus Galanthus quite early in their evolutionary history. This plant has a restricted habitat, is exceptionally rare, and thus rarely available on the market (Davis and Ozhatay, 2001).

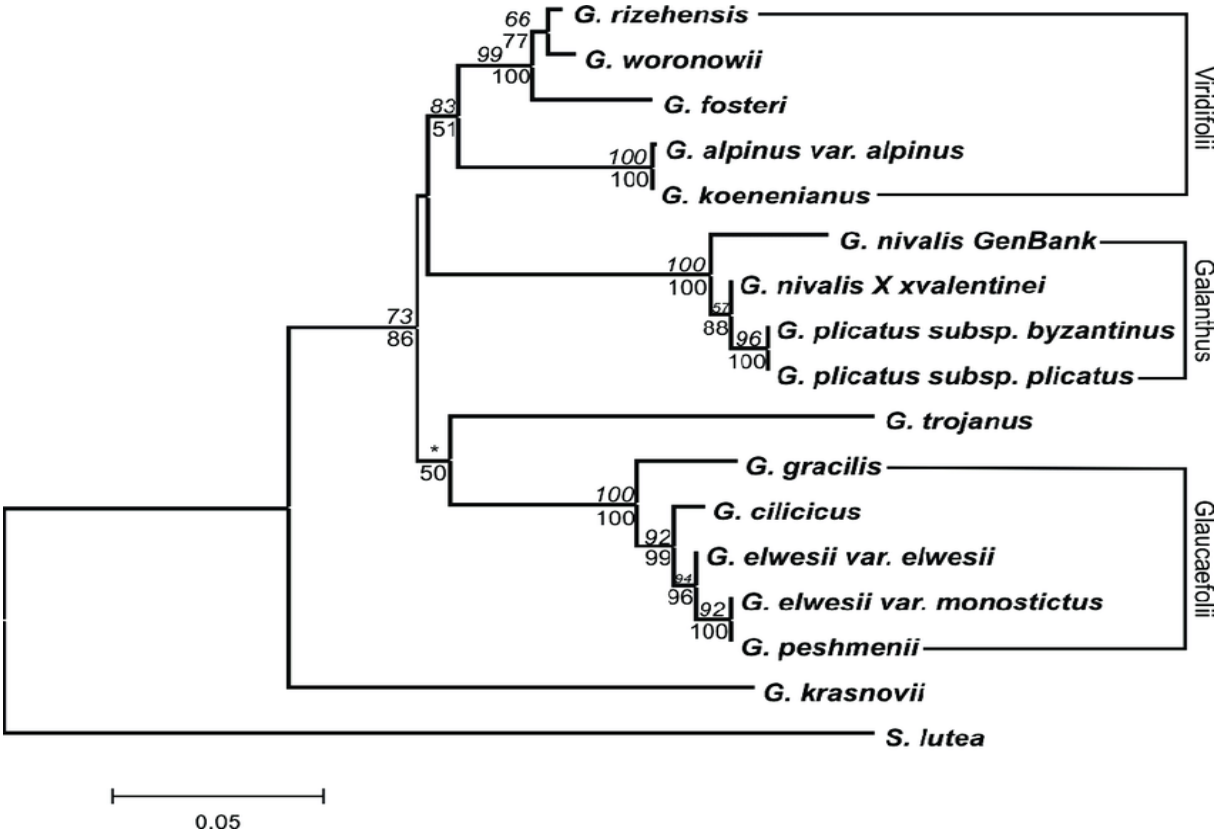


Figure 5. Phylogenetic tree of some *Galanthus* species grows in Turkey based on nuclear rRNA ITS sequence data (Taşçı et al., 2013).

Like other *Galanthus* species, *Galanthus trojanus* also produce alkaloid compounds, including tyramine, narcidine, stylopine, protopine, nicotinic acid, tyramine, and lycorine. They are valuable and are used in the pharmacological industry to produce drugs against some diseases. Among those alkaloid metabolites, lycorine is used in chemotherapy medicaments to treat cancer disease (Kaya et al., 2011).

Figure 6. *Galanthus trojanus* (Plants of Turkey, 2018).

Galanthus trojanus shares its morphological characteristics with *Galanthus rizehensis* and *Galanthus nivalis*. They flower from winter through spring, have applanate vernation, more or less linear leaves, and one mark at the apex of each inner perianth segment. (Davis and Ozhatay, 2001).

Galanthus trojanus differs from *Galanthus nivalis* by having a giant leaf and a short green mark at the tip of each inner perianth segment (Davis and Ozhatay, 2001).

Galanthus trojanus is distinguished from *Galanthus rizehensis* by its vast green leaf and the shape of its green inner perianth mark. *Galanthus rizehensis* has a U- or V-shaped inner perianth segment mark, but *G. trojanus* has a short, ill-defined V-shape mark (Davis and Ozhatay, 2001).

Galanthus rizehensis and *Galanthus nivalis* are shorter (scape length) than *Galanthus trojanus* and have smaller leaves and blooms than *G. trojanus* (Davis and Ozhatay, 2001).

Galanthus trojanus	*Galanhus nivalis*	*Galanthus rizehensis*

Figure 7. Various *Galanthus* species (Plants of Turkey, 2018)

1.1.2. Conservation Status of *Galanthus trojanus*

Human intervention endangers many *Galanthus* species in the wild (Davis, 1999), like *Galanthus trojanus*, *Galanthus cilicicus*, *Galanthus fosteri, and Galanthus panjutinii*. These species are generally endemic, but not all *Galanthus* species are threatened with extinction. A few species of *Galanthus* occur in many populations spread across extensive ranges. For example, *Galanthus elwesii, Galanthus plicatus*, *Galanthus alpinus,* and *Galanthus nivalis.*

There are three main threats to *Galanthus trojanus*. The first one, *Galanthus trojanus*, is renowned for its decorative(ornamental) value and conventional usage. That is why many *Galanthus trojanus* are exported annually and sold (unregulated *Galanthus* bulb trade). The second one, *Galanthus trojanus*, is a rare plant with a small geographic range that survives in an environment where agriculture competes for land usage. The third one, *Galanthus trojanus* is susceptible to climate change. Temperature changes negatively affect its existence and development. Because of these reasons, some *Galanthus* species were on the B1 list of critically endangered species by the IUCN in 2011 (International Union for Conservation of Nature, 2018).

Despite various conservation strategies and prohibitions, the existence of *Galanthus trojanus* is under threat. Because of these considerations, it is critical to use quick and regulated propagation methods rather than losing existing genotypes. At this point, the technique of plant

tissue culture can be an alternate practice. This technique prevents the loss of endangered plant species and allows several clones to be produced from a single plant. It is unnecessary to gather them from their wild areas to extract secondary metabolites from plants. Because tissue culture techniques can supply the raw product. (Hussain et al., 2012).

Some *Galanthus* species were regenerated in a tissue culture medium; the first study is organogenesis in *Galanthus woronowii* was produced by Popov and Cherkasov in 1984 using MS media, modified with the addition of 1 mg/1 NAA, BA, and Kinetin (Popov Yu and A., 1984). Then Zimmer and Girmen discovered the optimal propagation medium for bulblet production in *Galanthus* as bulb scale (Girmen and Zimmer, 1988). Also, *Galanthus woronowii, Galanthus transcaucasicus, Galanthus elwesii* Hook. were regenerated in plant tissue culture, but in vitro regeneration protocols have not been developed for *Galanthus trojanus* to date.

This research aims to create an efficiency and dependable in vitro regeneration system for *Galanthus trojanus* using two different explant sources which are with basal plate or not, as well as to evaluate the influence of plant growth regulators (PGR) on *Galanthus trojanus* in tissue culture.

1.2. Plant Tissue Culture

Plant tissue culture is a scientific method that utilizes the totipotency of plant cells in vitro on artificial media to regenerate entire or desired plant parts under aseptic and controlled conditions. It is used to improve plant quality, produce disease-free plants, produce secondary metabolites and bioactive chemicals in liquid cultures, and propagate plants in large numbers. This technique is called micropropagation and is rooted in the concept of plant cell totipotency, which means the ability of a cell to form the entire plant. During this process, cells' ability to arrange their metabolism, reproduction, and development is vital for plant regeneration (Hussain et al., 2012).

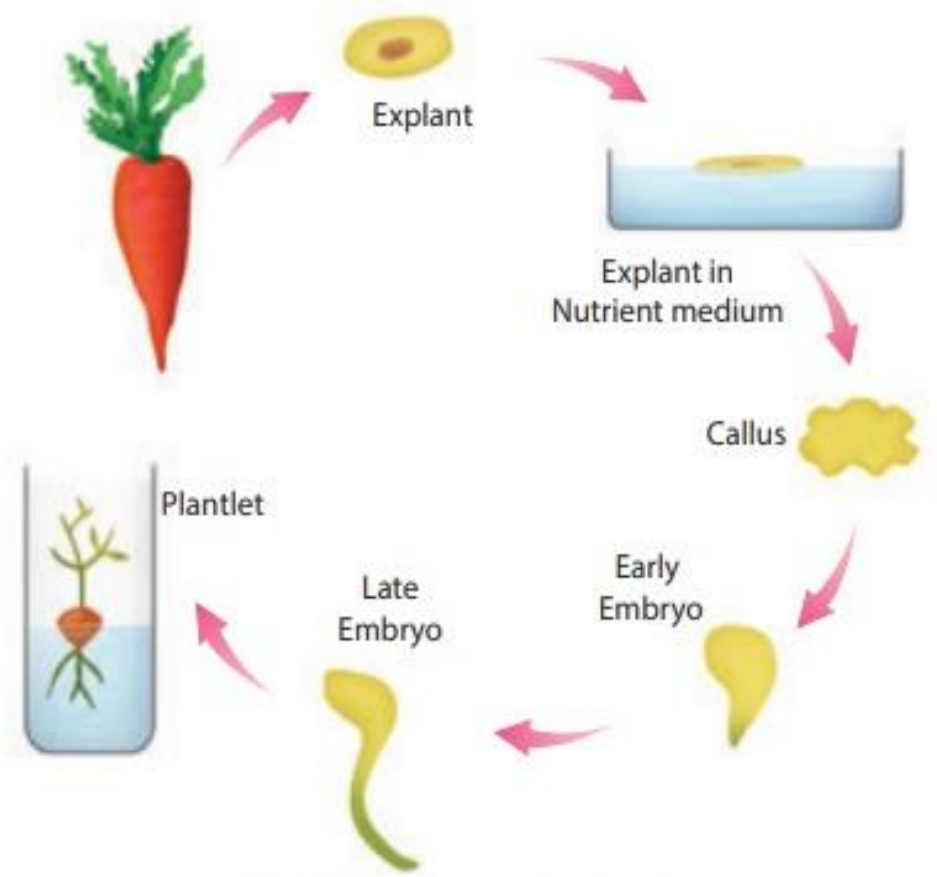


Figure 8. The plant cell's totipotent potential (Solutions, 2021).

The tissue culture media presents all the nutrients necessary for healthy plants to grow and develop. Various mediums are available for plant tissue culture, but White's medium, MS medium, B5 medium, N6 medium, and Nitsch's medium are the most commonly used (Hussain et al., 2012).

White's medium:

This is the oldest culture medium designed for root culture (Datta, 2019).

MS medium:

This first medium to promote organogenesis and plant was created by Murashige and Skoog (Murashige and Skoog, 1962).

B5 medium:

Initially intended for callus and cell suspension cultures, the B5 medium was developed by Gamborg (Phillips and Garda, 2019).

N6 medium:

Chu developed this medium, which is utilized for cereal anther culture and other tissue cultures (Thuzar et al.,2011).

Nitsch's medium:

Nitsch created this medium, which he regularly utilized for anther culture (Datta, 2019).

Due to its effectiveness with a diverse range of plants and culture systems, Murashige and Skoog media is the most popular medium for in vitro propagation of multiple plant species. The pH of the media is particularly significant since it influences plant development and plant growth regulators' functioning. It is set to a value between 5.4 and 5.9. Culturing can be done in both solid and liquid media. The content of the medium, especially the hormones and nitrogen source, has a significant impact on the initial explant development.

Table 2

Plant Tissue Culture Media Constituents

Components	**Amount (*mg l^{-1}*)**				
	White's	**Murashige and Skoog (MS)**	**Gamborg (B5)**	**Chu (N6)**	**Nitsch's**
Macronutrients					
$MgSO_4.7H_2O$	750	370	250	185	185
KH_2PO_4	-	170	-	400	68
$NaH_2PO_4.H_2O$	19	-	150	-	-
KNO_3	80	1900	2500	1830	950
NH_4NO_3	-	1650	-	-	720
$CaCI_2.2H_2O$	-	440	150	166	-
$(NH_4)_2.SO_4$	-	-	134	463	-
Micronutrients					
H_3BO_3	1.5	6.2	3	1. 6	-
$MnSO_4.4H_2O$	5	22.3	-	4.4	25
$MnSO_4.H_2O$	-	-	10	3.3	-
$ZnSO_4.7H_2O$	3	8. 6	2	1.5	10
$Na_2MoO_4.2H_2O$	-	0.25	0.25	-	0.25
$CuSO_4.5H_2O$	0.01	0.025	0.025	-	0.025
$CoCI_2.6H_2O$	-	0.025	0.025	-	0.025
KI	0.75	0.83	0.75	0.8	-
$FeSO_4.7H_2O$	-	27.8	-	27.8	27.8
$NA_2EDTA.2H_2O$	-	37.3	-	37.3	37.3
Sucrose (g)	20	30	20	50	20
Organic supplements					
Vitamins					
Thiamine HCI	0.01	0.5	10	1	0.5
Pyridoxine (HCI)	0.01	0.5	1	0.5	0.5
Nicotinic acid	0.05	0.5	1	0.5	5
Myoinositol	-	100	100	-	100
Others					
Glycine	3	2	-	-	2
Folic acid	-	-	-	-	0.5
Biotin	-	-	-	-	0.05
Ph	5.8	5.8	5.5	5.8	5.8

1.2.1. Compositions of Media

Plant nutrition and physiological activities require a variety of nutrients. So, these elements must be present in the culture medium for cultures to grow properly in vitro. Some of these elements and their functions in plants are given in Table 3.

.

Table 3

Some elements and their function in tissue culture medium

Element	*Function(s)*
Nitrogen	One of the building blocks of proteins, nucleic acids, and certain coenzymes. Required in large quantities.
Calcium	Play role in cell wall synthesis, membrane function, and cell signaling.
Magnesium	A cofactor for several enzymes and a constituent of chlorophyll.
Potassium	It is an important inorganic cation, adjust osmotic potential.
Phosphorus	Nucleic acid constituents and numerous intermediate in respiration and photosynthesis that are involved in energy transfer.
Sulfur	Component of certain amino acids (methionine, Cystine…).
Manganese	Cofactor for certain enzymes.
Iron	Component of cytochromes.
Chlorine	Participates in photosynthesis.
Copper	Involved in electron transfer reactions, Cofactor for some enzymes.
Cobalt	Component of vitamin B_{12}
Molybdenum	Component of certain enzymes (e.g., nitrate reductase), cofactor for some enzymes.
Zinc	Needed for chlorophyll biosynthesis, a cofactor for various enzymes.

All the nutrients necessary for healthy plants to grow and develop are present in the plant tissue culture medium. The medium is primarily made up of these constituents:

Table 4

Constituents of plant tissue culture media (Chimdessa, 2020)

a. Macronutrients	b. Micronutrients
c. Vitamins	d. Amino acids
e. Carbon sources	f. Plant preservative mixture
g. Gelling agents in the case of solid medium	i. Plant growth regulators

a. Macronutrients:

Macronutrients are elements that plants require in high concentrations (millimolar) for growth and development. Nitrogen, potassium, phosphorus, calcium, magnesium, and sulphur are the primary macronutrients, and nitrogen is the most important. It is required for plant growth in tissue culture since it is found in the structure of amino acids, nucleic acids, and proteins (Saad and Elshahed, 2012).

b. Micronutrients:

Micronutrients are elements that play a massive and essential role in plant cell and tissue growth but require extremely small amounts. The major micronutrients are Boron, Manganese, Iron, Zinc, Copper, Molybdenum, Cobalt, and Iodine. Also, iron is the most crucial microelement for plants, and plants need iron to synthesize chlorophyll and play a role in the conversion of energy during photosynthesis (Saad and Elshahed, 2012).

c. Vitamins:

Plants can produce vitamins that are essential for plant growth. On the other hand, some vitamins needed for plant tissue culture are fortified. These vitamins act as coenzymes and add to the plant tissue culture media for cell growth and development. Plants require them as catalysts in a variety of metabolic processes. When explants are cultivated in vitro, they act as constraints for cell growth and division. Thiamin (B1), Myo-inositol, Nicotinic acid, and Pyridoxine are some of these vitamins. Thiamin and Myo-inositol are required for the growth of all cells, and they are essential ingredients (Saad and Elshahed, 2012).

d. Amino acids:

Although most plants can synthesize the amino acids essential for proper development, some amino acid combinations may be added to the media since they are critical for forming cell and protoplast cultures. Plant cells get nitrogen from amino acids, which are easily absorbed by cells according to inorganic nitrogen sources. For example, the essential amino acid glycine is a significant component of plant tissue culture media. However, to control the oxidation of

phenolics and avoid tissue blackening, cysteine has been added to the media as an antioxidant (Chimdessa, 2020).

e. Carbohydrates:

A plant in tissue cultures cannot photosynthesize because there is no chlorophyll, poorly developed chloroplasts, limited CO2 in the culture vessel due to poor gaseous exchange, and lack of optimum light intensity. Thus, a carbon source must be added to the culture medium for the plant that occurs in various metabolic activities. The most common carbon source needed in tissue culture is sucrose at 2-5% (Saad and Elshahed, 2012).

f. Plant Preservative Mixture:

PPM is a preservative and biocide that destroys bacterial and fungal cells, stops spore germination, and can prevent endogenous contamination from explants at higher doses. Because of its heat stability, it can be autoclaved. PPM components enter bacterial or fungal cells and block their essential enzymes in central metabolic cycles such as the citric acid cycle and the electron transport chain. Also, PPM may prevent the entry of monosaccharides and amino acids into bacterial or fungal cells from the media (Staikidou et al., 2008).

PPM is a highly effective preservative/biocide that is exceedingly effective at optimal concentrations and does not hinder plant regeneration. We can now tackle this problem more efficiently thanks to PPM, which is cheaper and less risky than routinely used antibiotics. Also, it can inhibit contamination that is airborne, waterborne, and human contact-induced (Staikidou et al., 2008).

g. pH of Medium:

In tissue cultures, the ideal pH is between 5.0 and 6.0. After autoclaving, the pH usually drops by 0.3-0.5 units. While making the medium, the pH can be regulated to the needed ideal level before sterilization.

Plant cells in cultures stop developing when the pH is greater than 7.0 and lower than 4.5. A fresh medium must be prepared if the pH lowers throughout the plant tissue culture. Usually, above 6.0 and below 5.0 prevents appropriate form gelling in culture. (Huang and Murashige, 1977).

h. Gelling Agents:

The medium's hardness significantly influences the growth of explants in culture. Agar, agarose, and gellan gum are some of the gelling agents.

Among these, agar, a polysaccharide derived from seaweeds, is widely used in preparing plant tissue culture mediums. It allows the media to solidify. Also, agar is more appropriate according to other types. It generally melts between 60 and 100 ℃ and hardens around 45 ℃. The enzymes do not break down agar gels because they are unaffected by media components (Saad and Elshahed, 2012).

i. Plant Growth Regulators:

Plant growth regulators, a class of organic chemicals, play an essential role in culture medium because they control stem elongation, tropism, and apical dominance. Plant growth regulators (PGRs) are essential for identifying the developmental pathway of plant tissues in a culture medium. According to explant types and the research objective, the dose and kind of growth regulators or hormones are selected and determined (Singh et al., 2013). Plant growth regulators or hormones used in plant cell culture include auxins, cytokinins, gibberellins, and abscisic acid.

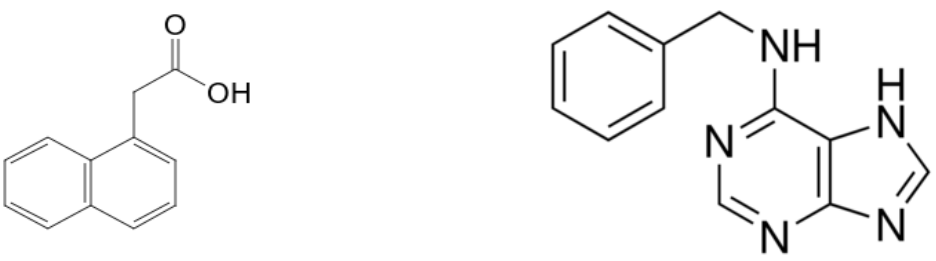


Figure 9. Structure of NAA and BAP plant growth regulator, respectively.

Auxins:

In cultures, it stimulates cell cycle and division, cell elongation, and callus production. Auxins encourage root development at low concentrations; callus formation occurs at high concentrations too. The common auxin types used in plant tissue culture are shown in table 3.4. (Bhatia, 2015).

Table 5

A list of plant growth regulators that have been employed in culture media

Growth Regulator (abbreviation/name)	*Chemical Name*
Auxins	
IAA	Indole 3-acetic acid
IBA	Indole 3-butyric acid
NAA	1-Naphthyl acetic acid
2, 4-D	2, 4-Dichlorophenoxy acetic acid
2, 4, 5-T	2, 4, 5-Trichlorophenoxy acetic acid
4-CPA	4-Chlorophenoxy acetic acid
NOA	2-Naphthyloxy acetic acid
MCPA	2-Methyl 4-chlorophenoxy acetic acid
Dicamba	2-Methoxy 3, 6-dichlorobenzoic acid
Piloram	4-Amino 2, 5, 6-trichlooropicolinic acid
Cytokinins	
BAP	6-Benzyl aminopurine
BA	Benzyl adenine
2İP (IPA)	N^6-(2-isopentyl) adenine
DPU	Diphenyl urea
Kinetin	6-Furfuryl aminopurine
Zeatin	4-Hydroxy 3-methytrans 2-butenyl aminopurine
Thidiazuron	1-Phenyl 3-(1, 2, 3-thiadiazol-5 yl) urea

Cytokinins:

In terms of chemistry, cytokinins are purine derivatives, specifically adenine. In the

culture media, cytokinins encourage the cell cycle, increase shoot development, and suppress root development. Cytokinins enhance protein and enzyme activity in tissues via promoting RNA synthesis (Bhatia, 2015). The most commonly used cytokinins are given in Figure 10.

The medium's ratio of auxins and cytokinins determined the short of culture. The formation of roots is generally induced by high auxin concentrations, while high cytokinin concentrations favor shoot regeneration. A balance of them provides callus formation, which is a mass of undifferentiated cells.

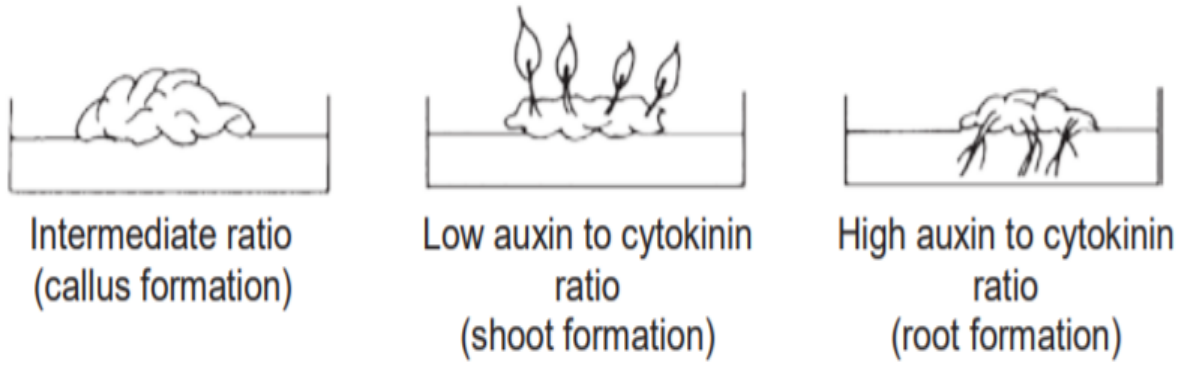


Figure 10. The rate of auxin and cytokinin.

Gibberellins:

As plant growth hormones, 20 distinct gibberellins have been recognized. They are commonly utilized in tissue culture and enhance the development of cultured cells, as well as callus formation and the elongation of dwarf plantlets. Based on the plant species, gibberellins can either promote or inhibit tissue cultures. They normally prevent the growth of accidental roots and shoots (Kumar and Loh, 2012).

Abscisic acid (ABA):

ABA can either promote or inhibit callus formation in cultures. It is mainly dependent on the species. ABA is a crucial growth regulator for embryonic induction (Bhatia, 2015).

1.2.2. Media Preparation:

The standard process for medium preparation includes preparing stock solutions (with concentrations ranging from (10x to 100x) with ultra-pure compounds and deionized water. Stock solutions can be kept frozen (in either plastic or glass vessels) and utilized whenever needed (Bhatia, 2015).

1.2.3. Preparing Media using Dry Powders:

The traditional method of preparing plant tissue media is time-consuming and labor-intensive. So, plant tissue culture mediums are now professionally manufactured and sold as dry powders on the market. To obtain the appropriate medium, mix the dry powder with a glass of deionized water. The other components, sugar, organic elements, and agar are added, the pH is regulated, and the medium is diluted to a final volume (Kumar and Loh, 2012).

1.2.4. Media Sterilization:

The culture media is sterilized in an autoclave for 20 minutes at 121°C and 15 psi. Then, filter-sterilized hormones and other heat-sensitive organic chemicals are introduced to the autoclaved medium (Kumar and Loh, 2012).

1.2.5. Choosing an Appropriate Medium:

Firstly, selecting a suitable medium for a specific plant culture system often starts with a recognized medium. According to the results, a fresh medium with the required properties regulators is prepared or developed. Also, the selection of growth regulators (auxins, cytokinins) is based on the culture system. In practice, 3-5 different growth regulator hormones are tested in various combinations and concentrations, and the best is chosen.

1.2.6. Stage of Plant in Tissue Culture:

Micropropagation

The initial stage in micropropagation is the choice of an explant from a healthy mother plant. Any portion of the plant can be used to get explants (leaf, apical meristem, bud, bulb, or root). To eliminate microbial contaminants, explants must be sterilized (Monthony et al., 2021).

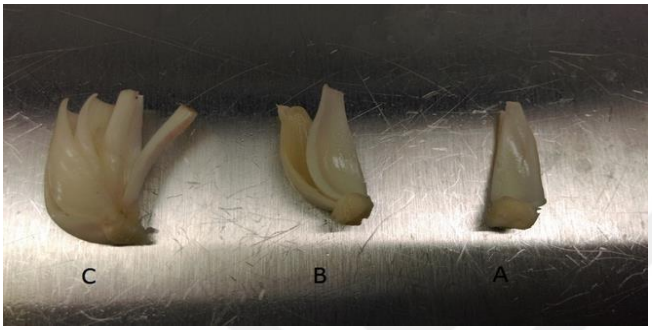


Figure 11. Three types of bulb explant (Babashpour-Asl et al., 2016).

Stage 0: Preparation of Donor Plant

If the mother plant is grown in ex vitro under appropriate conditions, it reduces contamination in the in vitro culture and chances the likelihood of success (Monthony et al., 2021).

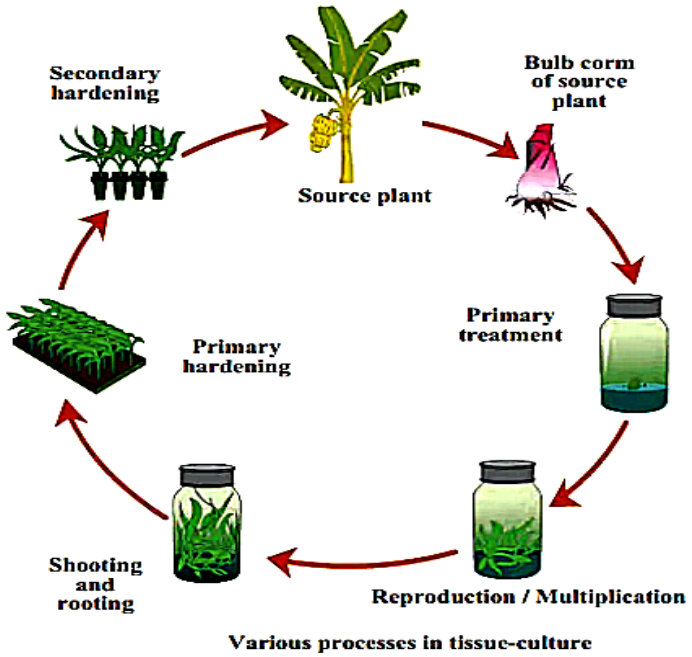


Figure 12. The tissue culture process**.**

Stage I: Initiation Stage

At this stage, sterilize the surface of the explant and insert it into the nutritional medium. PPM, bactericide, and fungicide can be used together or not according to explant type. The surface sterilization of explants with PPM, bactericide, and fungicide is an essential step in eliminating pollutants while causing the least amount of damage to explants. The most widely used disinfectants are sodium hypochlorite, calcium hypochlorite, ethanol, and mercuric chloride (Tilkat et al., 2009). The cultures are kept in a growth chamber (Monthony et al., 2021).

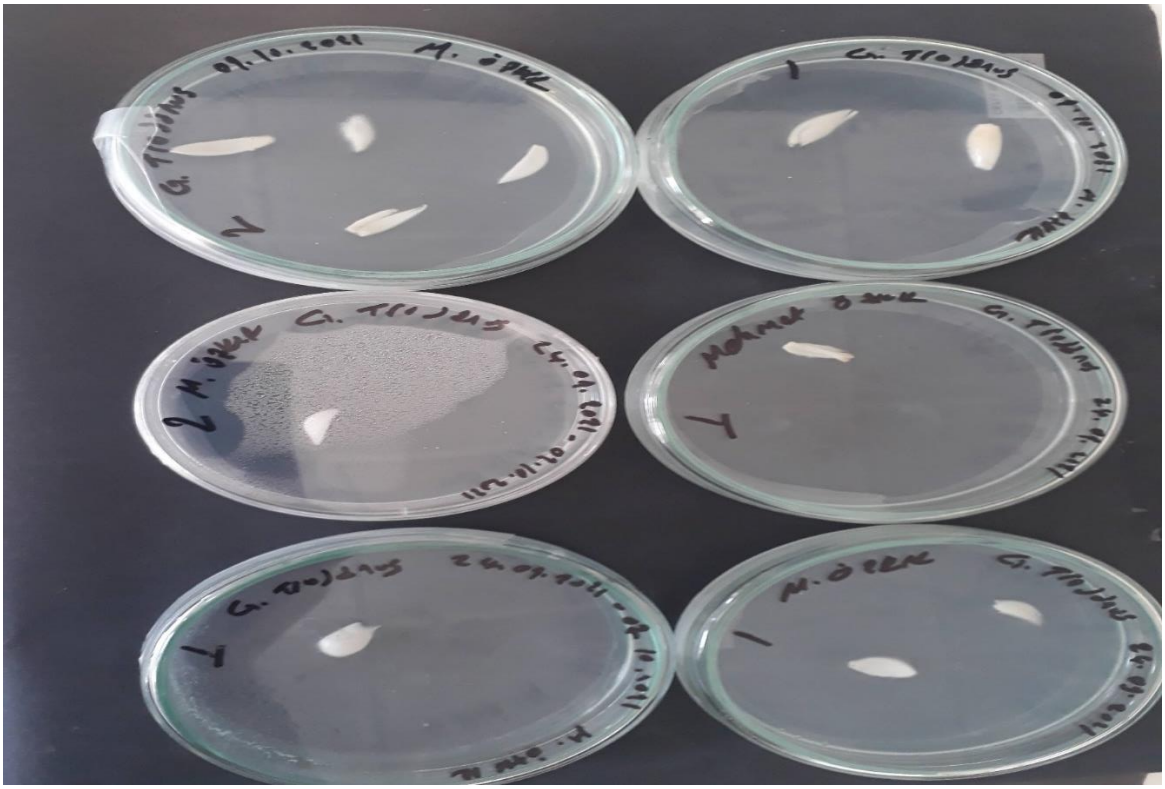


Figure 13. *Galanthus trojanus* placed in plant tissue culture media.

Stage II: Multiplication Stage

The objective of this phase is to produce more propagules. Until the desired number of plants is obtained, the number of propagules is repeatedly multiplied by the number of subcultures (Saini and Jaiwal, 2002).

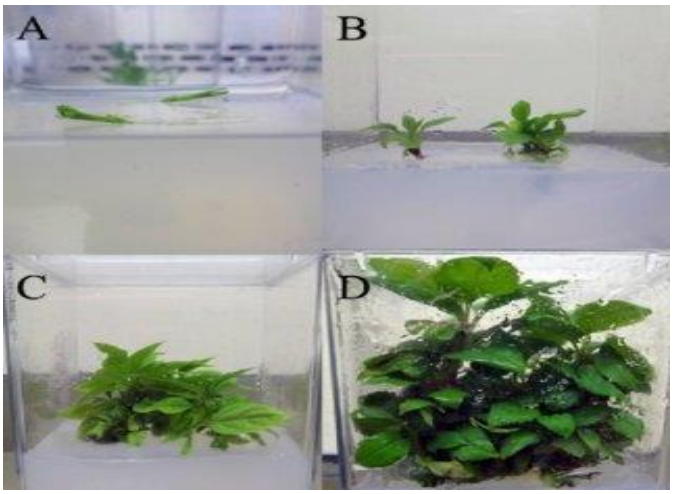


Figure 14. Explant multiplication (Zhu et al., 2018).

Stage III: Rooting Stage

The same culture medium employed for explant multiplication can be used for the rooting phase. In some circumstances, it may be required to alter the media composition, including nutritional alteration and growth regulator hormones, to promote rooting and establish vigorous root growth (Monthony et al., 2021).


Figure 15. Rooting Stage.

Stage IV: Acclimatization Stage

Plantlets should be adapting the climate change. Hardening (acclimatization) progresses

gradually from high to low humidity and from low to high light intensity. The plants are then placed in an appropriate object (sand, peat, compost) and gradually hardened under greenhouse conditions (Monthony et al., 2021).

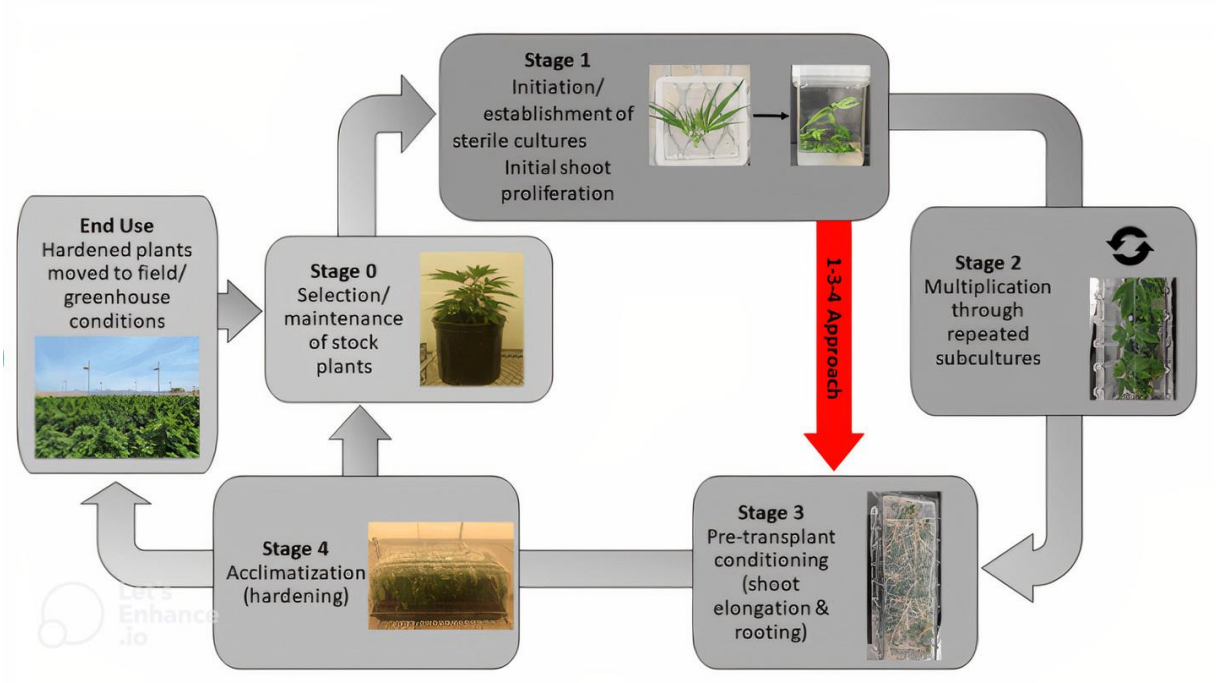


Figure 16. The five-stage of micropropagation (Monthony et al., 2021).

1.2.7. Types of Plant Tissue Culture:

There are many types of plant tissue culture which are depending on the cultured explants. These are given in table 6.

Table 6

Types of plant tissue culture

a- Cell culture	b- Callus culture
c- Organ culture	d- Embryo culture
e- Meristem culture	f- Pollen culture
g- Protoplast Culture	h- Seed culture

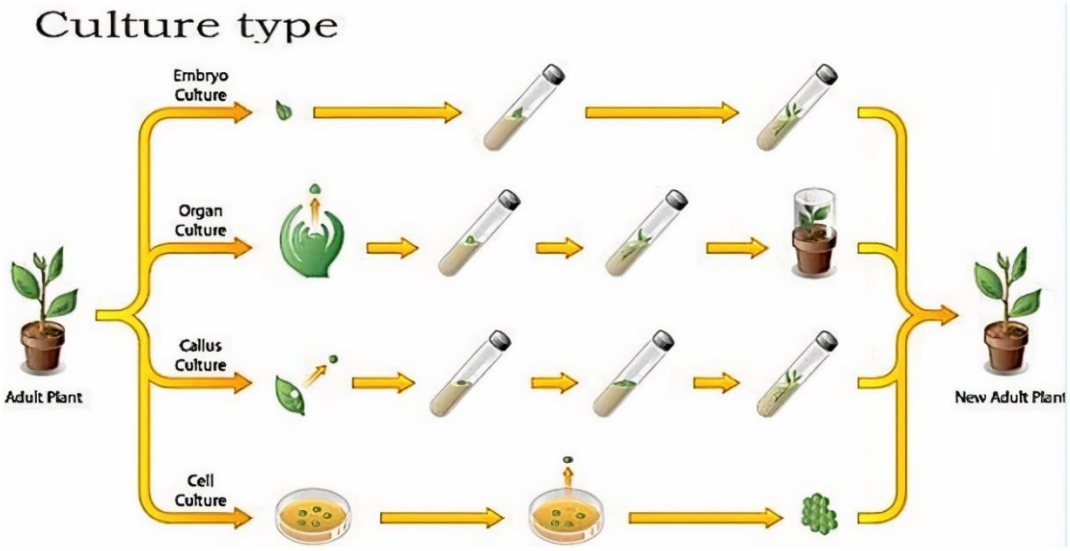


Figure 17. Selected some types of tissue culture (Kumar and Loh, 2012).

a. Cell Culture:

Cell culture is described as isolating individual cells from an explant tissue or callus and culturing this isolated single cell aseptically on a nutritional medium under controlled conditions. These cultures, known as cell suspension cultures, are carried out in dispersion media. Cell cultures are usually used in genetic transformation studies and the production of secondary metabolites (Bhatia, 2015).

b. Callus Culture:

Callus culture is the formation and maintenance of an unorganized mass of proliferative cells in glass tubes under controlled aseptic circumstances. For this purpose, artificial nutritional sources are used for isolated plant cells, tissues, or organs. It possesses the biological capacity to create a plant by developing proper roots, shoots, and embryos (Bhatia, 2015)

c. Organ Culture:

Organ culture refers to the cultivation of isolated plant organs. A nucellus, embryo, seed, root, endosperm, anther, ovary, ovule, meristem (shoot tip), or ovary can be used. İt can be either organized or unorganized. Organs select from a young and healthy part of the plant (Bhatia, 2015).

d. Embryo Culture:

Embryo culture is described as the aseptic extraction of embryos (at different developmental stages) from the bulk of maternal tissue of a mature seed or capsule, followed by in vitro culture in glass tubes with a nutrient-rich semisolid or liquid medium to develop immediately into plantlets. Embryo culture has been used to produce haploid plants (Bhatia, 2015).

e. Meristem Culture:

Meristem cultures are used to create plants that are mainly free from disease and contamination. Meristem culture is frequently favored because the absence of circulatory tissues limits disease contamination and transmission (Bhatia, 2015).

f. Pollen Culture:

Pollen culture is an in vitro method in which pollen grains are squeezed from intact anthers (preferably at the microscope stages) and cultured on nutritive media where microspores form without producing male gametes (Bhatia, 2015).

g. Protoplast Culture:

It is the aseptic cultivation of isolated protoplasts, naked plant cells surrounded by a plasma membrane but do not have a cell wall. Protoplast cultures may regenerate cell walls and dividing cells and develop and regenerate plants on suitable media. Also, it is necessary to cultivate hybrid plants (Bhatia, 2015).

h. Seed Culture:

In vitro cultivation of seeds is used to grow healthy plants or seedlings (Bhatia, 2015).

1.2.8. The importance of Plant Tissue Culture

In the present and future, the plant tissue culture technique is one of the most potential fields of industry. The areas range from cryopreservation of priceless germplasm to plant breeding. For the better nutritional value of primary agricultural plants, including trees, to manufacture medicinal compounds (seconder metabolite).

An effective in-vitro plant regeneration system is crucial for the success of all bioengineering techniques, including genetic engineering, haploid induction, and the investigation of somaclonal variation.

Plant tissue culture technologies are frequently used to cultivate and protect uncommon, endangered, and threatened species due to their high proliferation efficiency and minimal initial plant and space needs. No of the season or weather, a single explant can be replicated into many thousand plants under controlled conditions. Thanks to the plant tissue culture technique, any time or season of the year is suitable for plant production in tissue culture. (Hussain et al., 2012).

Plant tissue cultures offer great potential for producing many valuable bioactive molecules, called secondary metabolites, under controlled conditions (Varma, 2010). So, for decades, tissue culture has been used in the pharmaceutical industry. Plants' vast range of secondary metabolites is beneficial (and frequently crucial) in medicines, industrial materials, and food additives. Also, these essential metabolites are only produced in small amounts and frequently only in particular species. Each plant species will need a unique technique to examine optimal culture conditions. When the protocol is established, tissue culture can be used to produce thousands of plants with the same genetic profile. In this way, thousands of uniform plants are produced, and they have seconder metabolites. Many different medicinally important alkaloids are produced in plant cell and tissue cultures, such as shikonin and paclitaxel (Taxol).

Tissue culture techniques produce homozygous plants in a short time via protoplast, anther, and microspore cultures. Haploids are sterile plants with a single set of chromosomes that are transformed into homozygous diploids through spontaneous or stimulated chromosome doubling. The chromosome doubling recovers plant fertility, resulting in double haploids with the potential to become fresh breeding new cultivars (Hussain et al., 2012).

Plant cell culture has made tremendous progress. Plant cell culture may become increasingly important in its connection with transgenic plants in the years ahead. The capacity to speed up the standard multiplication rate can significantly benefit many places where crops are destroyed by disease or natural disasters. Once germplasm is stored in field genebanks, genetic resources are frequently lost. Slow growth in vitro storage and cryopreservation are being suggested as solutions to those problems that field genebanks face. They can be used in conjunction with field genebanks to provide a secure duplicate collection. So in the future, they will be able to access genetic resources for either basic traditional breeding programs or more complicated genetic transformation operations. It plays a significant role in agricultural growth and productivity (Hussain et al., 2012).

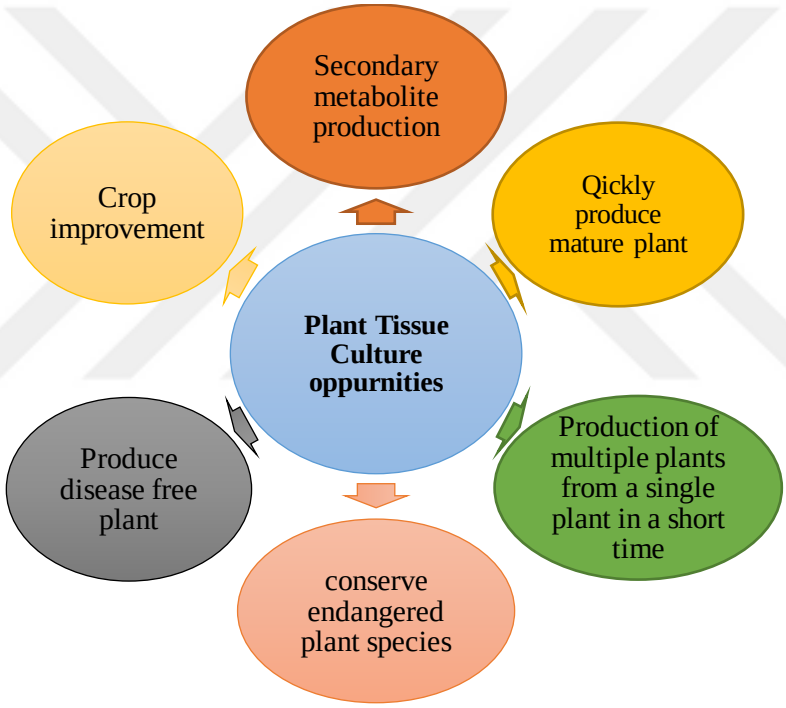


Figure 18. Some advantages of plant tissue culture

CHAPTER 2

PREVIOUS STUDIES

Galanthus, sometimes known as "Snowdrop," is the most popular flower bulb produced and marketed from Turkey's flora. *Galanthus* is a popular plant in Europe and other regions, and it is also widely recognized for its pharmacological activities. The plant's "galantamine" and "nivalin" alkaloids are primarily found in its bulbs. The alkaloid "galanthamine" is utilized as a raw ingredient in Polio vaccinations and treatments, and "nivalin" is used to treat some nerve illnesses (Tıpırdamaz et al., 1994).

Neriman Ozhatay, Andrew Byfield, and Margaret Johnson discovered an unnamed *Galanthus* (Snowdrop) in Turkey in 1994. The plants developed from seeds taken during this survey blossomed for the first time in 1997 at the Royal Botanic Garden in Kew, a district in southwest London. It quickly became evident that these plants could not be linked to any previously recognized snowdrop species. Then this plant got the name *Galanthus trojanus* and was published in 2001. The term relates to the growing territory of the Troas landscape that encircled the ancient city of Troy. The region is now part of the Turkish province of Çanakkale (Davis and Ozhatay, 2001).

Galanthus trojanus is a unique species because, according to new molecular phylogenetic studies, it split from other *Galanthus* species very early in the genus' evolutionary history. This snowdrop has a small habitat, is highly rare, and is almost non-existent on the market (Taşçı et al., 2013).

The interest in *Galanthus* grew soon after lectin and glycoprotein from *Galanthus* bulbs were identified as selective inhibitors of retrovirus and cytomegalovirus. Because chemical manufacture of such chemicals as galanthamine is not conceivable, extinction of snowdrop bulbs from nature and extraction of these is unavoidable (Tıpırdamaz et al., 1994). Thus, in 2011, *Galanthus trojanus* was included in the B1 list of critically endangered species by the IUCN (International Union for Conservation of Nature, 2001).

Because there is a significant drop in *Galanthus* species, it is necessary to use rapid and controlled growth methods rather than destroying existing ones. The primary causes are the

destruction of natural stocks, the extended life cycle, and the low replication rate of *Galanthus* species (Tıpırdamaz et al., 1994).

Tissue culture methods have been used on a vast range of *Galanthus*. These in vitro cultures were employed for mass plant propagation in the interest of species conservation, germplasm preservation, and alkaloid manufacturing. There is no need to extract plants from their native habitats to generate biologically active chemicals since tissue culture procedures could supply raw material for such purposes (Resetár et al., 2017).

The influences of explant type, various concentrations of sucrose, type, and different concentrations of auxin on bulblet production were examined to optimize conditions for micropropagation of *Galanthus transcaucasicus* Fomin. Finally, they developed a protocol for the regeneration system of *Galanthus transcaucasicus* Fomin (Babashpour-Asl et al., 2016).

The suitable explant type, nutrient media, growth regulators, and their concentrations on bulblet formation were determined for in vitro bulblet propagation of *Galanthus elwesii* Hook (Zencirkıran and Mengüç, 2004).

The micropropagation study overcame major obstacles such as explant contamination, hyperhydration, and slow bulb growth. Then demonstrate successful propagation of *Galanthus nivalis* via shoot cultures (Selby et al., 2005).

For the identification of lycorine in *Galanthus trojanus*, an accurate HPLC technique coupled with DAD detection was developed and validated (Kaya et al., 2013).

There is no study about the micropropagation of *Galanthus trojanus*. In the thesis frame, an efficient surface sterilization method was developed. Optimization of an effective and dependable in vitro regeneration system for *Galanthus trojanus by* using bulb explant examined and investigated the effect of plant growth regulators (PGR) on *Galanthus trojanus* in tissue culture.

CHAPTER 3

MATERIALS METHODS

3.1 Plant Material

Galanthus trojanus genotypes were provided by the Atatürk Horticultural Central Research Institute greenhouse, affiliated with the Ministry of Agriculture and Livestock, in the province of Yalova.

Figüre 19. Atatürk Horticultural Central Research Institute greenhouse.

The experiments of this thesis were carried out in the laboratories of Çanakkale Onsekiz Mart University, Faculty of Arts and Sciences, Department of Molecular Biology and Genetics.

3.2. Media Preparation

Media preparation is a crucial and fundamental stage in tissue culture. Because according to the types of explant or plant species, it should use different rates of compositions

of media such as concentration and rates of plant growth regulators.

3.2.1. Materials:

- Mutashige and Skoog (MS) medium (Powdered form)
- Sterilized distilled water
- Sucrose
- Plant agar
- PVP (Polyvinylpyrrolidone)
- PPM (Plant preservative mixture)
- BAP
- NAA

3.2.2. Apparatus:

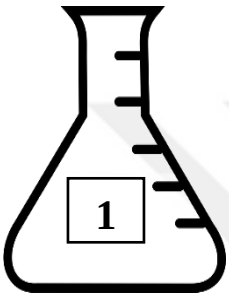
- Sodium hydroxide (NaOH)
- Hydrochloride acid (HCI)
- Glass petri dish
- Spatula
- Volumetric flask
- Autoclave
- Erlenmeyer flask
- Electronic balance
- Magnetic stirrer
- PH meter

For the preparation of 200 mL media,

- 0,88 gram MS powder
- 6 gram Sucroz
- 0.012 gram PVP

- 1.3 gram Plant agar
- 420 µl PPM
- NAA (4 different concentrations)
- BAP (4 different concentrations)

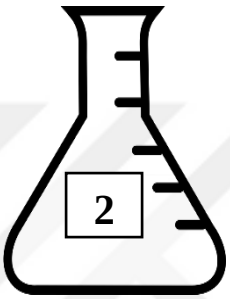
In this study, 200 mL of 4 different plant tissue culture media were prepared using the different concentrations of plant growth regulators (figure 20).



Control

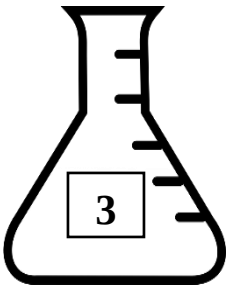
BAP : 0

NAA : 0



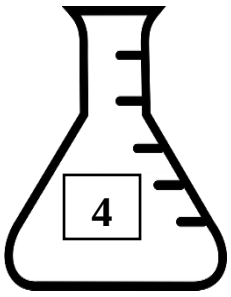
BAP: 0,5 mg/L

NAA: 0,1 mg/L



BAP : 1 mg/L

NAA : 0,2 mg/L



BAP: 2 mg/L

NAA: 0,4 mg/L

Figure 20. Types of media based on various concentrations of plant growth regulator hormones.

3.2.3. Calculation:

The concentration of our BAP and NAA plant growth regulators stock solutions were both 3 mg/ml. Firstly, 0.1 mg/L for 200 ml was prepared.

1 mg/ml = 1000 mg/L

X mg in X ml total solution ⟹ **C1 V1 = C2 V2**

C1: 3 mg/ml = 3000 mg/L , **V1**: ?

C2: 0,1 mg/L , **V2**: 200 ml = 0,2 L

C1 V1 = C2 V2

3000 mg/L x V1 = 0,1 mg/L x 0,2 L

V1 = 6, 66 µl ⟹ 6, 66 µl of NAA or BAP used to obtain 0,1 concentration of NAA or BAP in 200 ml solution.

Table 7

The concentration of NAA and BAP and the amount of them

Concentration	**Amount**
0,1 mg/L	6,66 µl NAA or BAP
0,2 mg/L	13,33 µl NAA or BAP
0,4 mg/L	26,66 µl NAA or BAP
0,5 mg/L	33,33 µl NAA or BAP
1 mg/L	66,66 µl NAA or BAP
2 mg/L	133,33 µl NAA or BAP

3.2.4. Methods:

In this study, four different media based on the concentration of plant growth regulators were prepared. Also, to understand the heat-stable of PPM, PPM was added only in one media and labeled before autoclaving.

I. Sterilized all material and apparatus by using the Autoclave machine.
II. 100 ml of sterilized distilled water filled in 4 different Erlenmeyer flasks.
III. 0,88 gr MS powdered medium is slowly added into each of 4 different Erlenmeyer flasks.
IV. 6 gr of sucrose is added into each Erlenmeyer flasks.
V. 0,012 grams is added into each Erlenmeyer flasks.
VI. Mixed each Erlenmeyer flask on Magnetic Stirrer.
VII. When flasks are mixed, pH is set at 5.8 by adding NaOH and HCI.

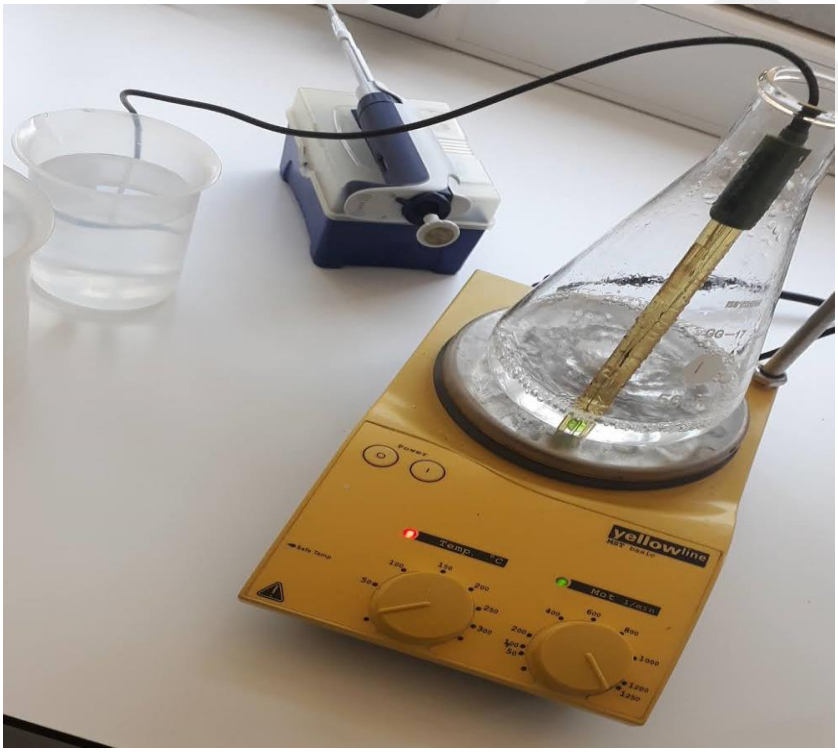


Figure 21. pH meter.

VIII. 1,3 gr agar technical was added to the four different Erlenmeyer flasks and slowly mixed.
IX. The media was prepared to be 200 ml by using a volumetric flask by adding 100 ml of distilled water.

X. 420 µl PPM is added to the label 2 Erlenmeyer flask before autoclaving and to other flasks after autoclaving.

XI. Cover the mouth of four different Erlenmeyer flasks that contain media with aluminum foil and autoclaved.

XII. 420 µl PPM is added to the label 1,3,4 Erlenmeyer flasks.

XIII. Added plant growth regulators in the laminar flow hood and labeled them.

1. MS basal (without plant growth regulators)
2. 0.5 mg/1 BAP + 0.1 mg/1 NAA
3. 1.0 mg/1 BAP + 0.2 mg/1 NAA
4. 2.0 mg/1 BAP + 0.4 mg/1 NAA

XIV. Four different flasks were gently mixed and waited for them to cool down a bit.

XV. The melting media is dispensed into the sterile glass petri dish in the laminar flow hood. Each tube is labeled.

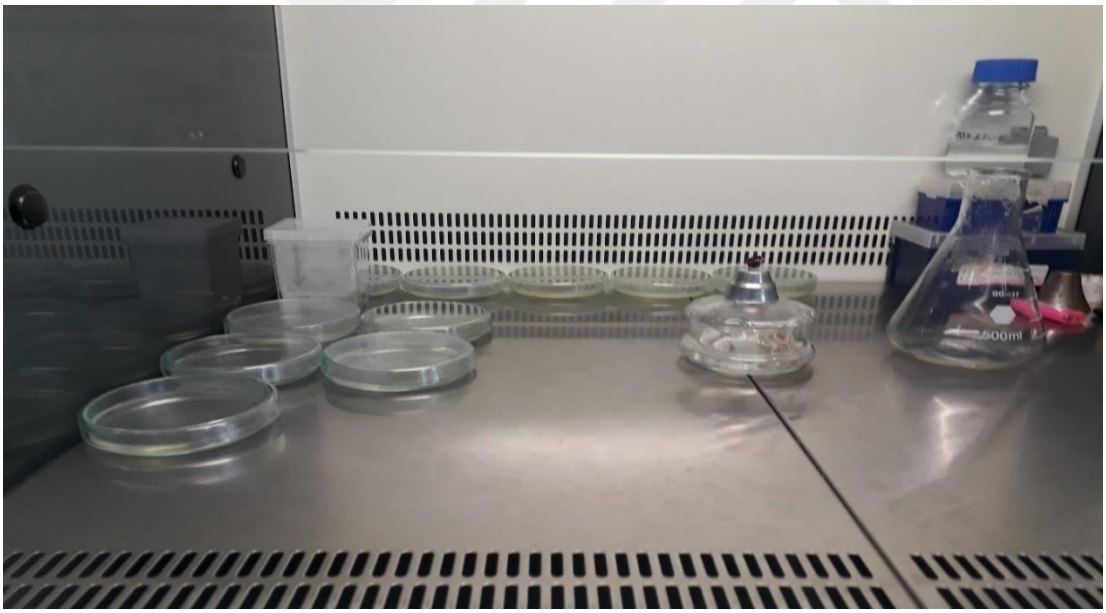


Figure 22. Media for plant tissue culture are prepared in the laminar flow hood.

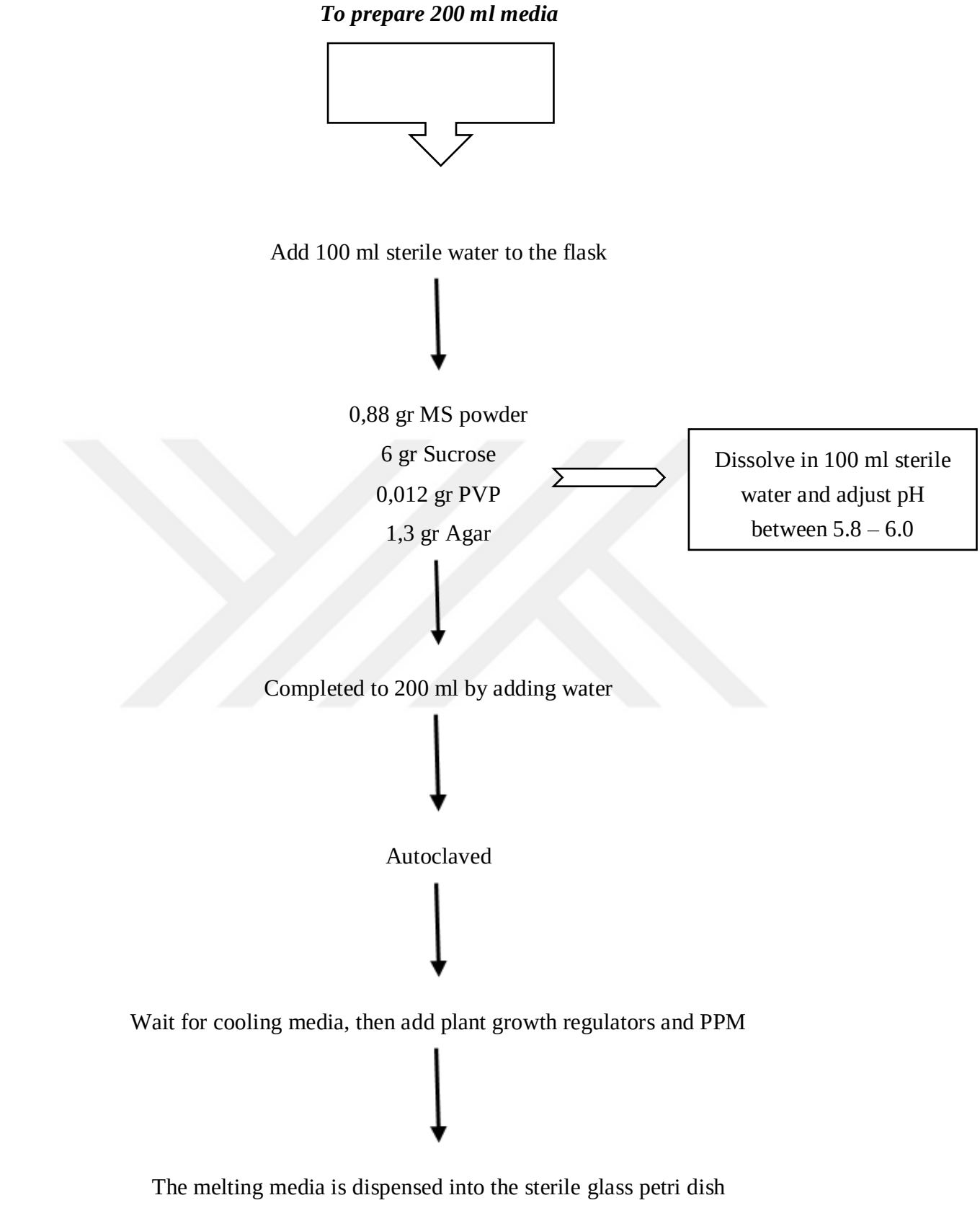


Figure 23. Summary of preparing plant tissue media.

420 µl PPM is added to the label 2 Erlenmeyer flask before autoclaved.	420 µl PPM is added to the label 1,3,4 Erlenmeyer flask after autoclaved.

Figure 24. Type of media based on PPM that was added before or after autoclaved.

3.3. Surface Sterilization and Preparation of Explants from *G. trojanus* Bulb Scales

To get rid of any bacteria or fungal spores present, plant material (bulbous) must first undergo surface sterilization to destroy all bacteria while causing no harm to the plant material..

3.3.1. Materials:

- PPM
- Sterilized distilled water
- 70% alcohol
- 75% in 100 ml Erlenmeyer flask for sterilization of forceps and scalpel
- 30% sodium hypochlorite (NaOCl)

3.3.2. Apparatus:

- Gloves
- Paper towel for drying explant
- Petri dishes for plant cutting
- Forceps
- Scalpel

- Beaker or container in which to wash the plant material
- 100 and 200 ml Erlenmeyer Flasks
- Parafilm

All material and apparatus were sterilized by using the autoclave machine at 15 psi for 30 minutes.

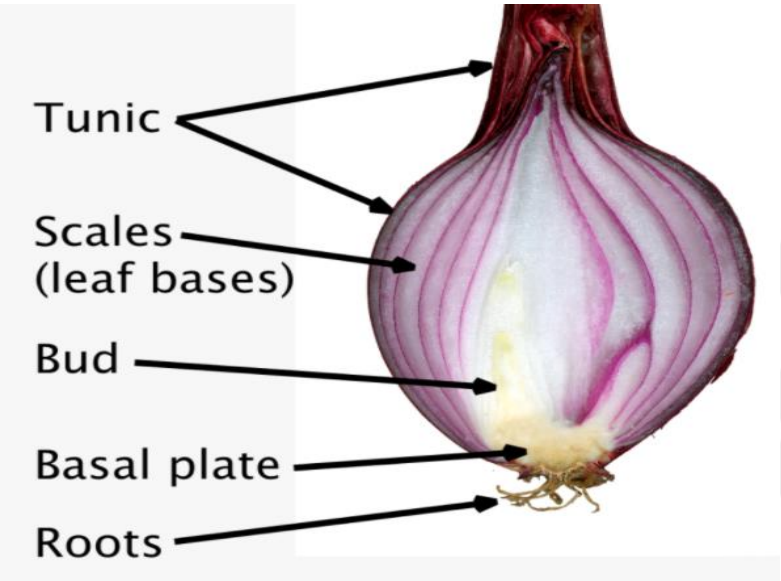


Figure 25. cross-section of onion bulb.

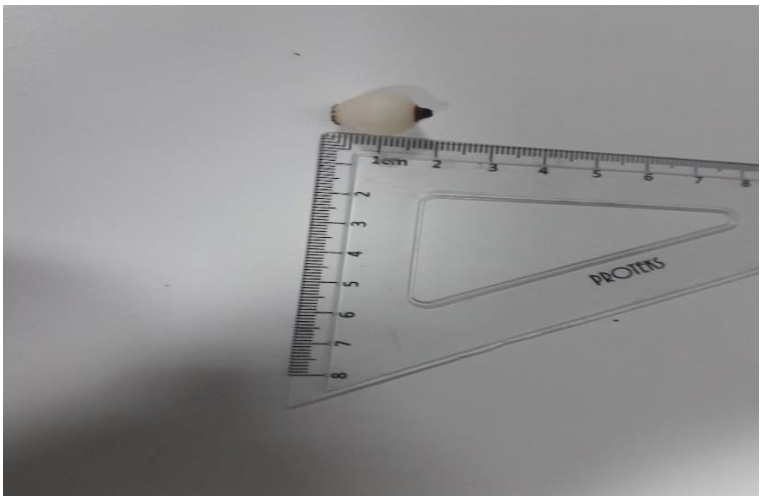


Figure 26. The size of the bulb is measured with a ruler.

3.3.3. Methods:

In sterilization methods, two separate experiments were performed to observe and determine the treatment of explant with PPM before inoculating the plant tissue culture media.

Experiment-1:

I. Firstly the thin layer of basal bulb tissues was cut away, trimmed, and cleaned, Then washed for about 30 minutes to obtain white and healthy tissues.
II. The healthy and white bulb stayed under tap water for about 1 hour.
III. The bulbs were surface sterilized by rinsing 70% ethyl alcohol for 3 min.
IV. Afterward, the bulbs were soaked in 30% sodium hypochlorite for about 30 minutes.

Figure 27. The bulbs were soaked in 30% sodium hypochlorite for about 30 minutes.

V. Finally, the bulb was rinsed with sterile distilled water three or five times.
VI. The bulb is placed on the blotting paper and allowed to dry for 10 minutes.

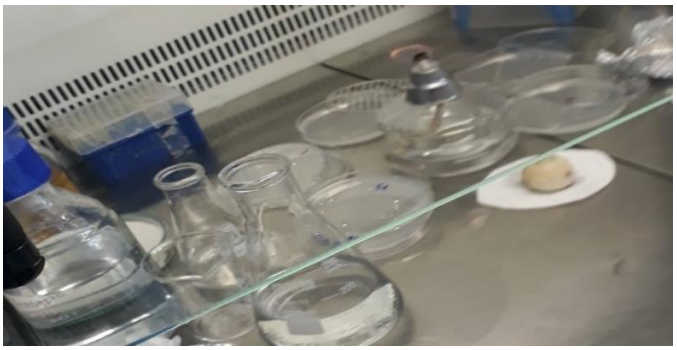


Figure 28. The bulb is placed on the blotting paper.

VII. The scalpel and forceps are burnt by soaking them in 75% ethanol, followed by flaming and cooling to sterilize

Figure 29. The scalpel and forceps are being sterilized.

VIII. *G. trojanus* bulbs with a diameter of 2 cm were sliced vertically into four equal sections into a sterile glass petri dish using a scalpel and forceps.

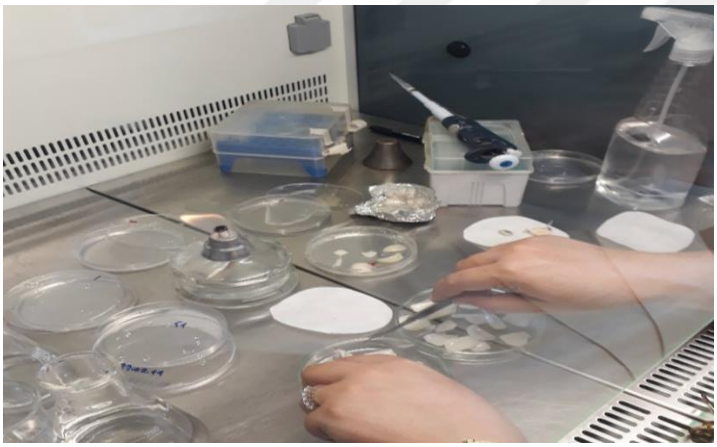


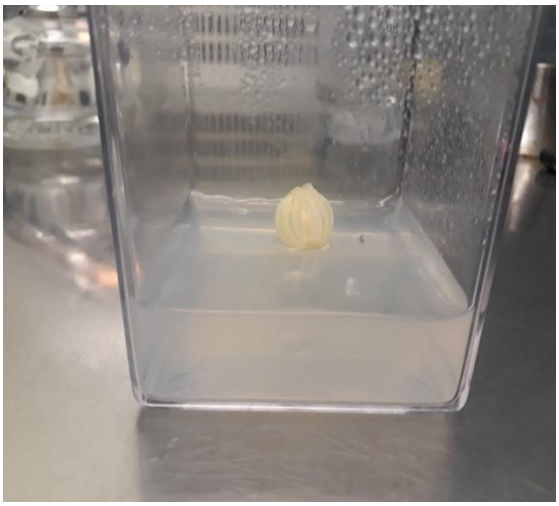
Figure 30. The bulb is sliced into the sterilized glass petri dish.

IX. Explants were formed by separating these pieces into single scales, twin scales, and tri-scales.

X. Explants were soaked into a beaker containing 4% (v/v) PPM (Plant Preservative Mixture) solution and shaken at 100 rpm for 9-12 hours.

XI. After 9-12 hours, explants treated with PPM are inoculated directly into the glass petri dish without rinsing.

XII. All explants are inoculated into the glass petri dish.



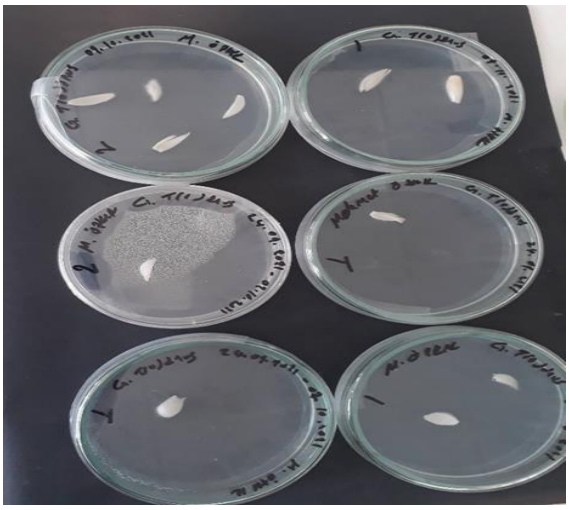


Figure 31. Explant in plant tissue culture media.

XIII. Parafilm is used for sealing glass petri dishes to avoid contamination.

XIV. The cultures were kept in a growth chamber at 25 ºC with a 16-hour photoperiod provided by LED lamps.

Figure 32. The cultures were kept in a growth chamber.

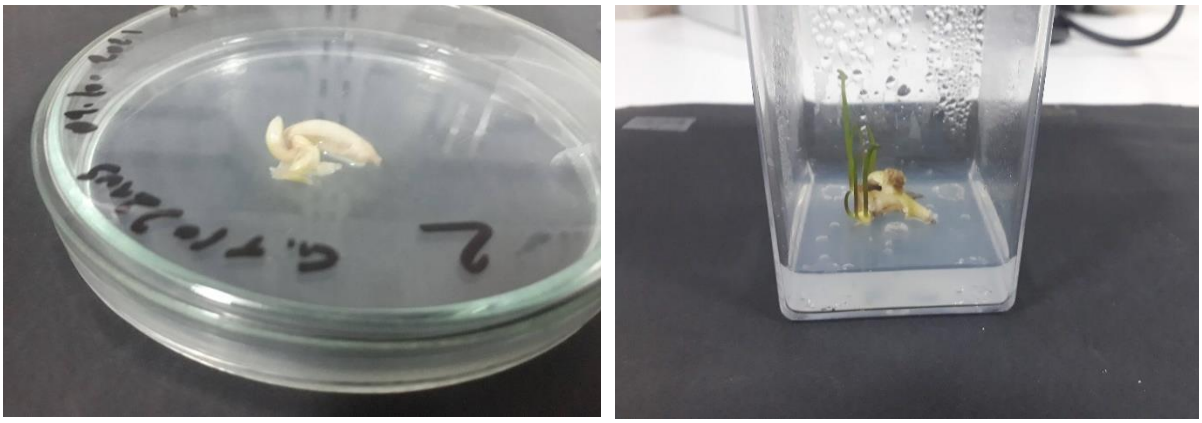


Figure 33. Explant in plant tissue culture media.

Experiment-2:

I. Firstly, the thin layer of basal bulb tissues were cut away, trimmed, cleaned, and washed for about 30 minutes to obtain white and healthy tissues.
II. The healthy and white bulb stayed under tap water for about 1 hour.
III. The bulbs were surface sterilized, rinsing in 70% ethyl alcohol for 3 min.
IV. Afterward, the bulbs were soaked in 30% sodium hypochlorite for about 30 minutes.
V. Finally, the bulb was rinsed with sterile distilled water three or five times.
VI. The bulb is placed on the blotting paper and allowed to dry for 10 minutes.
VII. The scalpel and forceps are burnt by soaking them in 75% ethanol, followed by flaming and cooling.
VIII. *G. trojanus* bulbs with a diameter of 2 cm were sliced vertically into four equal sections into a sterile glass petri dish by using a scalpel and forceps.
IX. Explants were formed by separating these pieces into single scales, twin scales, and tri-scales.
X. Then, explants are inoculated into the sterile glass petri dish directly.
XI. Parafilm is used for sealing glass petri dishes to avoid contamination.
XII. The cultures were kept in a growth chamber at 25 ºC with a 16-hour photoperiod provided by LED lamps.

3.4. Statistical Analysis:

The statistical analysis of the data was interpreted by performing the “One Way Anova” in the IBM SPSS Statistics 21.0 package program.

CHAPTER 4

RESEARCH FINDINGS

4.1. Explant Selection

In plant tissue culture applications, plants and plant organs regenerate in vitro by being affected by plant growth regulators (auxin and cytokinin) applied internally and externally, explant type, and culture conditions.

Bulb scales, particularly from the basal region at the connection with the base plate, and already differentiated buds have been the most beneficial explants for bulbous plants.

For *Galanthus* species, bulb sections generally are used as explants despite the difficulties in sterilization because they have high regenerative capacity. In vitro propagation of *Galanthus elwesii* demonstrates that the *Galanthus* bulbs have a high regeneration capacity according to the ovary, flower stalk, and petiole (Girmen and Zimmer, 1988). In the frame of these findings, bulb scales were used as explant.

In this study, two different bulb explant types were used: explants have a basal plate and no basal plate.

Explants with and without basal plates were transferred to 4 different MS nutrient media based on plant growth regulators, shown in table 8. As a result of the observations made at the end of 5 months in these media, bulblets with or without basal plate displayed different formation capacities. 21 of 48 explants with basal plates were grown in both media except the media that did not contain plant growth regulators, while explants without basal plates were not grown in four media. These results showed that explants without basal plates have no regeneration capacity. Also, the bulb's basal plate vertically inserted 2 or 3 mm into the media was most appropriate for growing.

Table 8

The impact of plant growth regulators on explants containing and not containing basal plates

Plant growth regulators		Number of explants with basal plate (48)		Explant without basal plate (16)	
BAP	NAA	Grow	Not Grow	Grow	Not Grow
0	0	0	12	0	4
0.5 mg/L	0.1 mg/L	9	3	0	4
1.0 mg/L	0.2 mg/L	6	6	0	4
2.0 mg/L	0.4 mg/L	6	6	0	4
Total		21	27	0	16

For explant with the basal plate

Explants were not regenerated in the hormone-free medium.

Media containing 0.5 mg/L BAP and 0.1 mg/L NAA plant growth regulators were determined as the most suitable medium for regenerating bulb explants with the basal plate.

Also, media containing 1.0 mg/L BAP, 0.2 mg/L NAA and 2.0 mg/L BAP, 0.4 mg/L NAA plant growth regulators showed similar properties for regeneration of bulb explants.

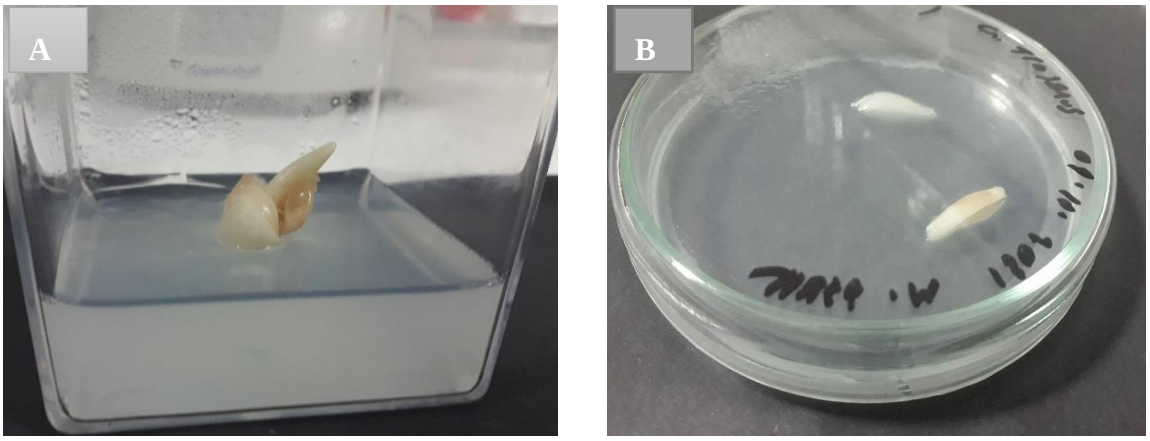


Figure 34. A. Explant with basal plates B. Explant without basal plates

4.2. Effect of PPM and NaOCl Sterilization

Surface sterilization of the explant is possibly the most critical treatment prior to culture initiation. Because in vitro conditions give bacteria and fungi an excellent growth environment, inadequate sterilization inhibits tissue culture investigations. Therefore, the surface-sterilization method seeks to eradicate all bacteria that can quickly develop in vitro conditions without damaging the explant's survival and regeneration potential.

Galanthus trojanus have a small bulb; thus, the influential surface sterilization of its bulb explants to a level appropriate for micropropagation is challenging. So, before slicing chip explants, sterilizing methods included 30% NaOCl treatments of entire bulbs in conjunction with 70% ethanol. This approach was performed with 4% PPM treatments directly administered to bulb chip explants.

However, the concentration and kind of disinfectant can affect explant vitality and renewal capability. For example, sodium hypochlorite (NaOCl) harms explant viability at high concentrations, but it is also ineffective for explant sterilization at low doses (Yildiz and Celal, 2002). In vitro propagation protocols have been reported previously for some *Galanthus* species. These reports showed safe quantities for using NaOCl; some examples were in vitro propagation of *Galanthus nivalis* and *Galanthus elwesii.* They were sterilized separately with 50% NaOCl for 20 minutes (Selby et al., 2005). While in vitro propagation of *Galanthus transcaucasicus,* in sterilization step Fomin was used with 10% NaOCl for 20 minutes; after that, 2.5% NaOCl was used for 10 minutes (Babashpour-Asl et al., 2016), and while sterilization of *Galanthus nivalis* Fomin was used with 30% NaOCl for 30 minutes (Zencirkıran and Mengüç, 2004). In this study, 30% NaOCl was preferred because *Galanthus trojanus's* bulbs were small, and the regeneration capacity of small explants typically was lower according to large explants. Because of this, 30% NaOCl was used, with no risk of further reducing the regeneration capacity.

PPM is also the most effective sterilant. Nevertheless, it significantly reduced tissue viability and growth based on species. For *Galanthus*, tissues sterilized with 4-5% PPM were unaffected by its viability and regeneration capacity (Staikidou et al., 2006).

The sterilization of bulbs is more complicated than other parts of the plant. Because bulbs have complicated structures with zones between scale leaves. However, the treatment of bulb chip explants with PPM was highly effective. Because there are a few reasons. Firstly, any additional sterilization procedure with a different mechanism of action is likely to kill any residual microorganisms. Secondly, bulbs have complicated structures with zones between scale leaves or deep base plate cracks that sterilants cannot easily reach. However, microbes are found in bulbs' tissues, and they strongly associate with them. Furthermore, soil particles are attached to bulbs and are difficult to remove without damaging some tissues; thus, contamination of culture is hazardous. To overcome these problems, bulbs were cut in half longitudinally, and sterilization was improved (Goddard, 2000). Explants soak into a beaker that contains 4% (v/v) PPM for about 9 hours. Moreover, infection hidden deep within bulbs will be dispersed among explants on the scalpel or in rinsing water during bulb chip explant cutting.

Initially, the media were prepared, but PPM was not added. Also, the explants were sterilized with standard protocol except for keeping them in PPM solution for 9-12 hours. After a week, all petri dishes were contaminated, and we could not prevent contamination without using PPM.

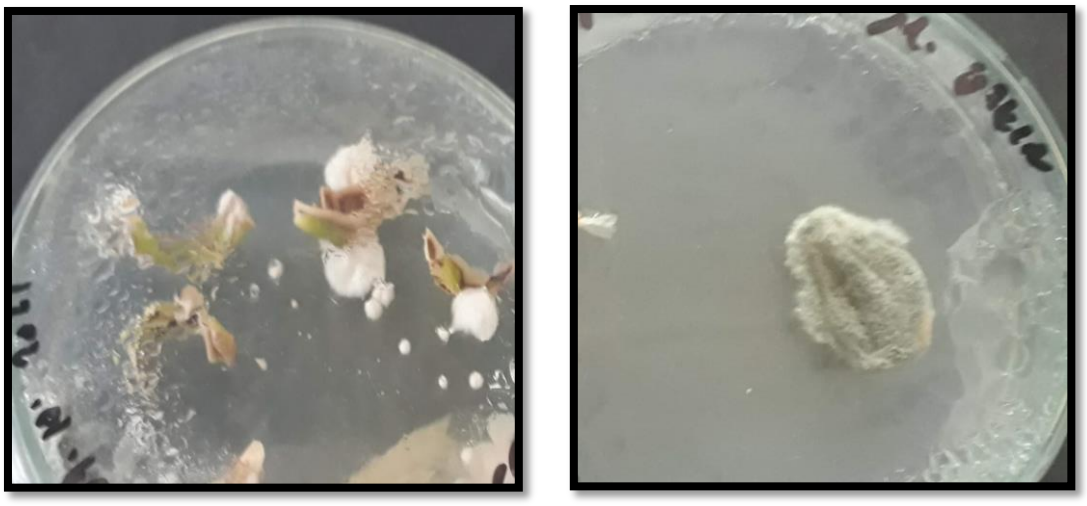


Figure 35. Contamination of explant and culture media.

After that, the media were prepared by adding PPM, and the explants were sterilized with typical protocol except for keeping in PPM solution for 9-12 hours. Even though PPM was added, the media still got moldy and took a long time.

Figure 36. Contamination of explant.

In the last trial, media which includes 2.1 ml/L PPM, was prepared, and the explants were sterilized with the combination of 4% PPM solution. The result was terrific, and the number of moldy media was deficient. So, these conclusions show that PPM should be used to prevent media contamination.

Figure 37. The media and explants were not contaminated.

The results showed us that the use of 30% NaOCl for 30 minutes and 4% PPM for 9 hours had no risk of reducing the regeneration capacity. Also, it has been observed that PPM is resistant to heat.

4.3. The Effect of Different Concentrations of NAA and BAP on Bulblet Production of *G. trojanus*

The interactions between auxin and cytokinin are considered essential regulators of organ development in cultured tissues. The axillary proliferation and shoot proliferation happen when the ratio of auxins to cytokinins is low; embryogenesis, callus initiation, and root initiation happen when the ratio is high (Sivparsad and Gubba, 2012).

A maximum shoot regeneration of 75% was observed on MS medium with 0.5 mg/L BAP with 0.1 mg/L NAA (Table 9). The other culture media (1.0 mg/L BAP with 0.2 mg/L NAA or 2.0 mg /L BAP with 0.4 mg/L NAA) showed low regeneration percentages (50%, 41.7%, respectively). The Plant growth regulator free culture media have no regeneration capacity. All these results showed that MS medium supplemented with the combination of 0.5 mg/L BAP and 0.1mg/L NAA was the most suitable treatment medium for shoot formation.

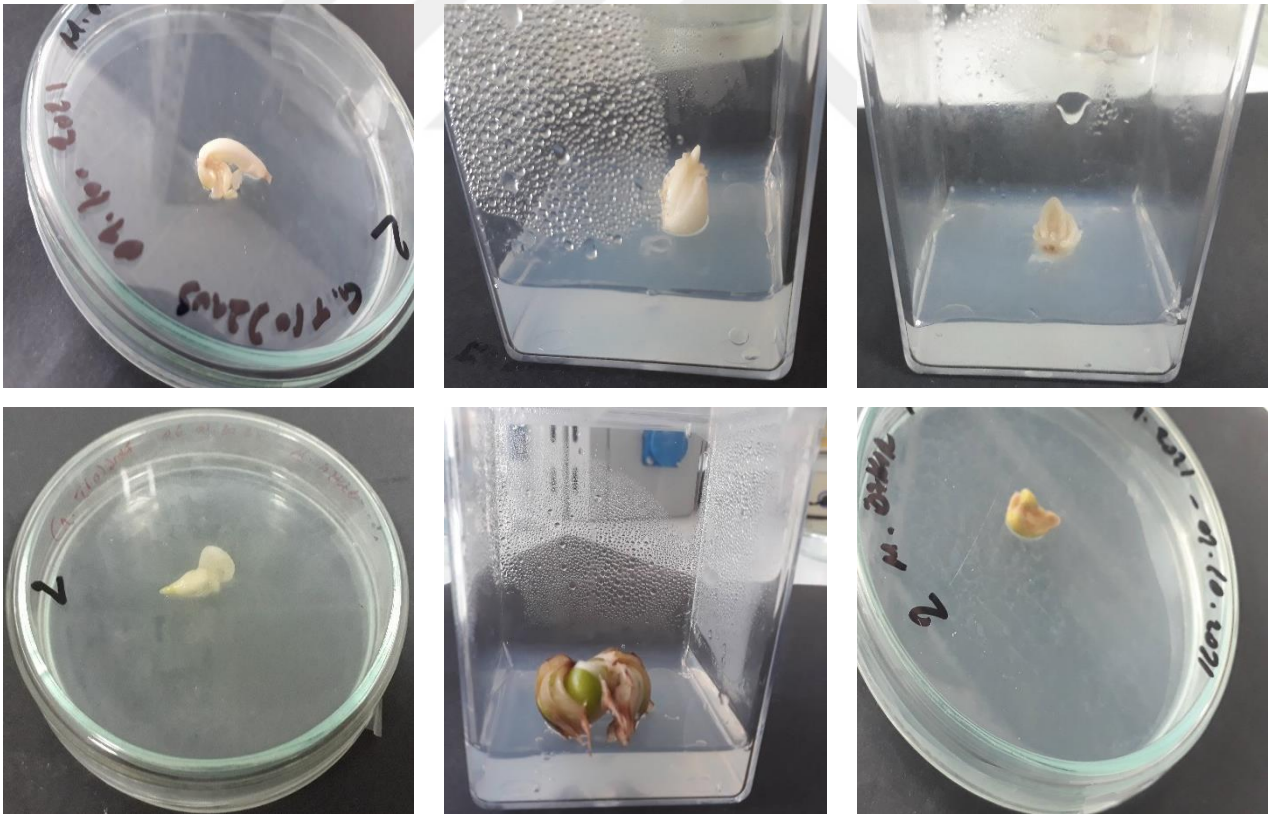


Figure 38. Regeneration of *Galanthus trojanus* bulb explants in a 60-day period.

Table 9

The impact of plant growth regulators on shoot formation in *Galanthus trojanus*

Plant growth regulators		Shoot regeneration (%)	Mean number of shoots per explant
BAP	NAA		
0	0	0	0
0.5 mg/l	0.1 mg/l	75 a	3.33 a
1.0 mg/l	0.,2mg/l	50 b	1.33 b
2.0 mg/l	0.4mg/l	41.7 b	1.08 b
Mean		41.7	1. 43

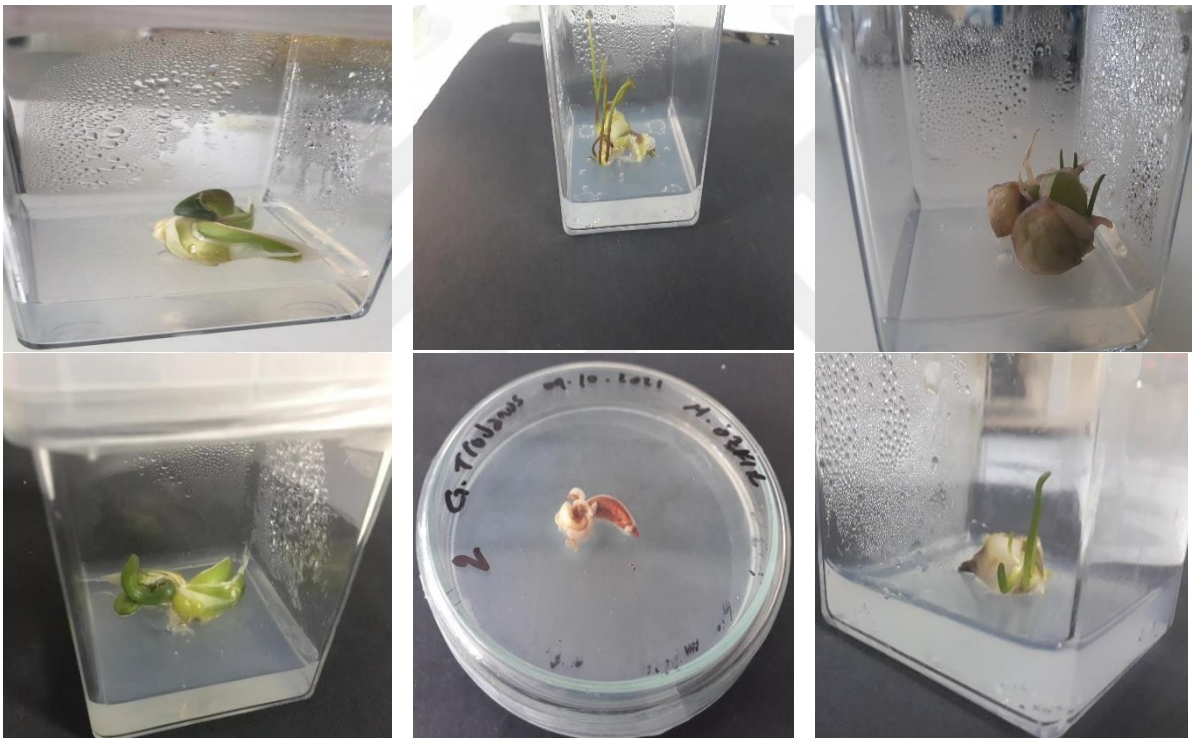


Figure 39. Regeneration of *Galanthus trojanus* bulb explants in a 120-day period.

However, maximum shoot and callus formation of 50% was recorded in the MS medium with 2.0 mg/L BAP plus 0.4 mg/L NAA (Table 9). The other culture media (those containing 0.5 mg/L BAP plus 0.1 mg/L NAA or 1.0 mg /L BAP plus 0.2 mg/L NAA) showed low regeneration percentages (16.6 %, 25%, respectively). The culture media containing no hormone have no regeneration capacity. All these results gave us that 2.0 mg/L BAP interacts with 0.4 mg/L NAA media was determined as the most effective medium for shoot and callus formation.

Table 10

The impact of plant growth regulators on shoot and callus formation in *Galanthus trojanus*

Plant growth regulators		Shoot and Callus formation together (%)
BAP	NAA	
0	0	0
0.5 mg/l	0.1 mg/l	16.7 a
1.0 mg/l	0.,2mg/l	25.0 a
2.0 mg/l	0.4mg/l	50.0 b
Mean		22.9

Values are the means of 48 explants and mean values in each column followed by a different lower-case letter are statistically different at $P \leq 0.05$ by Duncan's multiple range test.

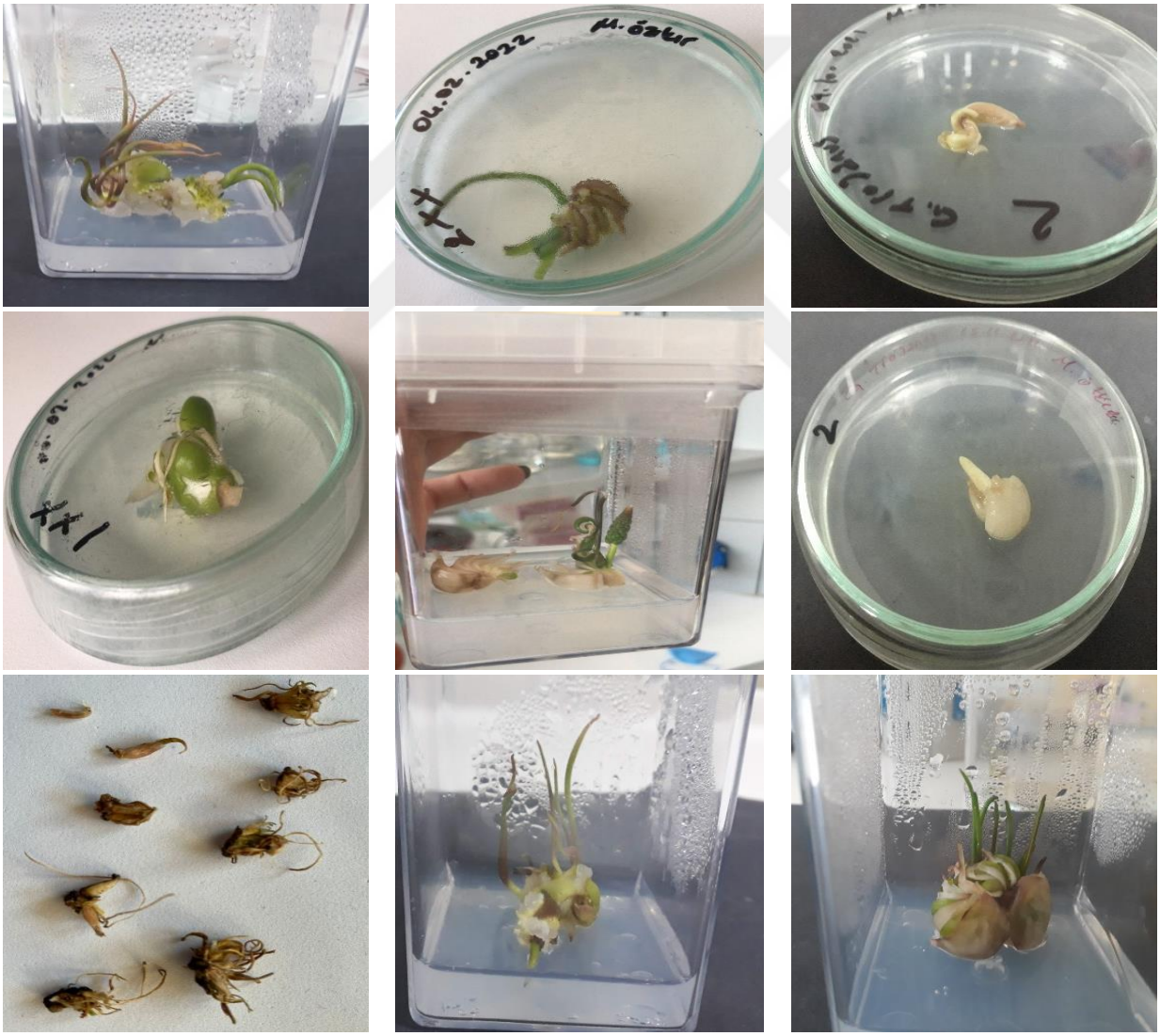


Figure 40. Shoot, bulb, and callus formation in *Galanthus trojanus* bulb explants in a 150-day period.

CHAPTER 5

RESULTS AND RECOMMENDATIONS

Galanthus trojanus are economically significant due to their ornamental value and use as landscaping plants. Because they have colourful and attractive flowers, also, they are the most popular flower bulb collected and exported from Turkey's flora. Its harvested and exported to many European countries. This gives hundreds of households of small-scale farmers in impoverished rural areas a significant source of financial income.

Besides its ornamental value, *Galanthus trojanus* is a medicinal plant since it produces pharmacologically active alkaloids with various structures and exciting biological functions. Stylopine, protopine, nicotinic acid, tyramine, and lycorine are some of these alkaloids (Kaya et al., 2011). Among these compounds, lycorine displays antiviral activity and is cell growth and division stopper. So, it is a potential chemotherapeutic drug to cure some cancer diseases. These substances serve a similar purpose (Berkov et al., 2012).

However, in progress of time, *Galanthus trojanus* and other *Galanthus* species have gradually lost because of their economic value for ornamental and pharmaceutically property and growth in agricultural areas. Also, they are gathered and removed unconsciously from nature, leading to a sharp decrease in their quantity. Thus, they also face extinction threats due to a lack of suitable propagation systems (Zencirkıran and Mengüç, 2004).

So, in 2011, *Galanthus trojanus* was on the B1 list of critically endangered species by the IUCN. Also, it is in the Annex-II list according to the Convention Regulating the International Trade of Endangered Wild Plant and Animal Species (International Union for Conservation of Nature, 2018).

Despite various conservation strategies and prohibitions, the existence of *Galanthus trojanus* is under threat. To prevent the extinction of *Galanthus trojanus* and other *Galanthus* species and increase the number of individuals in species to obtain many more pharmacological compounds they produce, we should establish in vitro regeneration system protocol, also known as plant tissue culture.

Thanks to the studies in which in situ and ex situ conservation programs are carried out in a complementary manner, much more successful results can be obtained to determine the species that are in danger of extinction and ensure the continuation of their generations. In vitro techniques for ex situ conservation programs are an essential option for the conservation and reproduction of endemic and endangered plant species.

The plant tissue culture method, an in vitro technique, is the leading, innovative, and invaluable tool to conserve medicinal and endangered plants from probable destruction and can produce multiple plants from a single plant. Especially for the manufacture of biologically active alkaloid compounds, there is no need to harvest plants from their native habitats because tissue culture processes can provide the raw material. So, in vitro propagation methods (tissue culture) are one of the fastest and most controlled methods of propagating *Galanthus* species.

The natural propagation speed of *Galanthus trojanus* is comparatively low, which often hinders the widespread cultivation of these plants. This problem may be resolved through tissue culture techniques. *Galanthus trojanus* plantlets, produced with micropropagation methods, could be used to produce pharmacologically important alkaloid metabolites.

According to our knowledge of the literature, no micropropagation study of *Galanthus trojanus* has been carried out. So, this current study is the first report on tissue culturing of *Galanthus trojanus* using different bulb explants and established an efficient protocol. The effect of different levels of NAA and BAP on bulbs of *Galanthus trojanus*, explant type, and sterilization methods were determined.

This study's first step was sterilizing the *Galanthus trojanus's* bulb. The sterilization of bulbs is more complicated than other parts of the plant because bulbs have complicated structures with zones between scale leaves. Nevertheless, treating bulb chip explants with PPM was highly effective, and sterilization efforts to obtain sterile *Galanthus trojanus* bulb explants have been successful.

In determining protocol, firstly, the bulbs were washed by hand for about 30 min and stayed under tap water for about 1 hour. Afterward, the surface was sterilized by rinsing in 70% ethyl alcohol for 3 min, then soaking in a 30% w/v) sodium hypochlorite for about 30 min, followed by five washes in sterile deionized water. Finally, explants were formed from bulbs

and soaked into a beaker that contained 4% (v/v) PPM solution and shaken at 100 rpm for 9-12 hours. Also, the MS medium was supplemented with PPM at about 2.1 ml/L to prevent contamination. The results showed us that the use of 30% NaOCl for 30 minutes and 4% PPM for 9 hours had no risk of reducing the regeneration capacity. Also, it has been observed that PPM is resistant to heat.

Due to their great regenerative capacity, bulb section explants are the most commonly used, despite the problems in sterilizing. In this study, two different bulb explant types were examined for their regenerating potential based on whether or not they had basal plates. The average regeneration capacity of explant with basal plate is 43.7% in four different media based on different concentrations of plant growth regulators. In contrast, an explant without a basal plate has no regeneration capacity (Table 8).

Since the difference in auxin (NAA) and cytokinin (BAP) ratios changes the shape of the tissue structure, a high cytokinin/auxin ratio supports shoot formation, a high auxin/cytokinin ratio supports root formation, and equal auxin and cytokinin ratios support callus formation. After five months of culture in bulb explants that have the basal plate, as observed from statistical analysis, shoot formation occurred in three different media containing plant growth hormones. But hormone-free media do not contribute to the regeneration of explants. However, the highest number of shoots were produced on MS medium supplemented with 0.1 mg/L NAA and 0.5 mg/L BAP after five months (Table 8).

Besides that, when the increased concentration of BAP and NAA combination synergistically improves the formation of callus and shoots together. According to our statistical analysis in table 10, the supplemented MS medium with 2.0 mg/l BAP and 0.4mg/l NAA concentrations is the most appropriate for callus and shoot formation, which is about 50%. The results of the other MS mediums with 0.5 mg/L BAP and 0.1 mg/L NAA or 1.0 mg /L BAP with 0.2 mg/L NAA displayed similar formation capacity of callus and shoot formation, which are 16.7 %, 25%, respectively.

11 of 48 explants found in four types of our MS medium were formed, both shoot and callus (table 11). Also, no explant regeneration was recorded in the MS medium, which does not contain plant growth regulators (BAP and NAA). Thus, for the explant to regenerate, there must be a hormone in the media (Table 8, Table 9).

Table 11

The percentage of shoots and callus formation together

Shoots and Callus formation together (48)	Frequency	Percent
No formation	37	77.10
Formation	11	22.9
Total	48	100,0

Consequently, MS medium supplemented with the combination of 0.5 mg/l BAP and 0.1mg/l NAA was the most suitable treatment medium for shoot formation, which was about 75% (table 9). It is seen that *Galanthus trojanus*, which has an important place in our country's geophytes, is suitable for reproduction by tissue culture. This, in the commercial production of *Galanthus trojanus*, can be a good source for rapid disease-free plant production in larger areas and can also be used to obtain alkaloid compounds.

In micropropagation studies, shoots are transferred to rooting media containing high auxin to form a complete plant. Species require NAA or BAP hormones to support rooting. MS nutrient media containing different concentrations of BAP and NAA were used for the rooting of the shoots obtained in previous studies. In our study, various attempts were made to ensure rooting and acclimization of *Galanthus trojanus* shoots which are *in vitro* micropropagated, but the desired results could not be obtained. For root formation, high auxin (NAA) with low or none cytokine (BAP) has been reported to have a stimulating effect (Tıpırdamaz, 2003). Before our study, for root formation of *Galanthus ikariae,* When 0.5 mg/L NAA was added to the media, there was a substantial change in rooting rate compared to the control media (0.1 mg/L, 0.01 mg/L), and it provided the greatest rooting value (without using cytokine). Also, it was stated that the amount of sucrose did not have a significant effect on root formation. (Tıpırdamaz, 2003). Also, *Galanthus woronowoii* shoots which are grow in MS culture transferred the MS media that contain 1.0 mg/L auxin and give best result in formation of root (Öztürk, 2021).

In our study, 2 different MS nutrient media were used in terms of growth regulator types

and concentrations for root formation. The first media which is include 0,5 mg/L NAA with 0,1 mg/L BAP and the second media which is include only 0,5 mg/L NAA. But no rooting was observed in shoot and bulb explants transferred to rooting media.

There could be several reasons why our rooting work failed. Some of these, are the amount of other composition MS medium, genetic difference of *Galanthus trojanus and* its adaptation properties, difference concentration of auxin and cytokine combination, and environmental conditions. So, our study is going on to overcome this problem and ensure rooting. Also, further direct and indirect tissue culture investigations on *Galanthus trojanus* organs are required to synthesize bioactive alkaloids.

No study has been found on the micropropagation of the endemic and endangered *Galanthus trojanus* species by tissue culture. Contrary to traditional production, it is possible to obtain fast and many healthy plants with in vitro techniques. Studies such as revealing the ecological requirements of the endangered species' distribution areas and life cycle characteristics are essential in determining conservation strategies. In the areas where the species are distributed, severe destruction has been observed due to human activities, and the species is endangered. For this reason, the results obtained in the research will be the first data for the micropropagation of *Galanthus trojanus* and will constitute both a source and one of the conservation methods for making the necessary conservation plans for the species. In addition, the research is vital in terms of contributing to tissue culture studies to be carried out on other endemic and endangered plants and enabling biotechnological research on these plants in the future.

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