REPUBLIC OF TURKEY
ERCIYES UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES DEPARTMENT OF AGRICULTURAL SCIENCES AND TECHNOLOGIES

INDUCTION OF CALLUS FROM LEAF EXPLANTS OF BLACK MULBERRY (*Morus nigra* L)

Prepared By
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Supervisor
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M. Sc. Thesis

Ağustos 2024
KAYSERİ

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SCIENTIFIC ETHICS SUITABILITY

I, Samuel OBEDGIU, hereby declare that this piece of work is the result of my research and that this work has never been submitted anywhere for any degree. All the sources of information used were obtained in accordance with academic ethics and have been duly acknowledged.

Samuel OBEDGIU

COMPLIANCE WITH GUIDE

This master's thesis writes up on the topic "Induction of callus from leaf explants of black mulberry (*Morus nigra* L)" was prepared with accordance Erciyes University's graduate thesis writing directive

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Samuel OBEDGIU

Erciyes University, Graduate School of Natural and Applied Sciences
Master of Science Thesis, August 2024
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ABSTRACT

This research involved callus culture established from leaf explants of *M. nigra.* The various concentration of MS (Murashige and Skoog) media, Lloyd & McCown Woody Plant Basal Media and blueberry media were examined and the impacts of different types of auxins including Indole-3-acetic acid (IAA), 2,4-Dichlorophenoxyacetic acid (2,4-D), a-naphthaleneacetic acid (NAA)] and cytokinin including Zeatin, Thidiazuron (TDZ), 6-benzyl amino purine (BAP), and silver nitrate at a concentration of 0.0 2.5, 5.0, 7.5, 10.0,20.0 µM, were examined to identify the optimal media for induction and maintenance. Half-strength (½) MS media supplemented with 7.5µM TDZ produced the most optimal callus induction, healthy and friable callus. Callus induction response was also achieved on full-strength Lloyd & McCown Woody Plant Basal Media with 10 µM TDZ. Full strength blueberry media supplemented with 20µM zeatin while ½ Lloyd & McCown Woody Plant Basal Media and half-strength blueberry media didn't induce any callus. Full strength WPM media with NAA, 2,4-D and BAP also induced callus from the leaf explants while regardless of the concentration IAA. Finally, AgNO3 didn't support any callus induction from the leaf explants of *M. nigra*

Keywords: callus cultures, growth curve, medicinal plant.

INDUCTION OF CALLUS FROM LEAF EXPLANTS OF BLACK MULBERRY (*M. nigra* L)

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ÖZET

Morus nigra meyve türünün yaprak eksplantlarından kallus kültürü çalışılmıştır. MS (Murashige ve Skoog) ortamı, Lloyd & McCown Woody Plant Basal Ortamı ve yaban mersini ortamının optimal konsantrasyonu incelenmiş ve İndol-3-asetik asit (IAA), 2,4-Diklorofenoksiasetik asit (2,4-D), a-naftalenasetik asit (NAA)] ve Zeatin, Thidiazuron (TDZ), 6-benzil amino purin (BAP) gibi sitokininlerin farklı konsantrasyonları (0,0 2,5, 5,0, 7,5, 10, ve 20 µM) çalışılmıştır. 7,5 µM TDZ ile desteklenen yarı güçlü (½) MS ortamıen uygun kallus oluşumunu sağlamıştır. 10 µM TDZ içeren tam güçlü Lloyd & McCown Odunsu Bitki Bazal Ortamında da kallus oluşumunu teşvik etmiştir. 20µM zeatin ile desteklenirken tam kuvvetli yaban mersini ortamı, ½ Lloyd & McCown Woody Plant Basal ortamı ve yarı kuvvetli yaban mersini ortamı herhangi bir kallus oluşturmamıştır. NAA, 2,4-D ve BAP içeren tam güçlü WPM ortamı kullanılan IAA konsantrasyondan bağımsız olarak da yaprak eksplantlarından kallus oluşumunu sağlamıştır. Son olarak, AgNO3, M. nigra'nın yaprak eksplantlarından kallus oluşumunu teşvik etmemiştir.

Anahtar Kelimeler: kallus kültürleri, büyüme eğrisi, şifalı bitki.

CONTENTS

INDUCTION OF CALLUS FROM LEAF EXPLANTS OF BLACK MULBERRY (*M. nigra* L).

ABBREVIATIONS

BAP : 6-benzylamino purine

MS : (Murashige and Skoog) nutrient media

TDZ : Thidiazuron

WPM, Lloyd & McCown : Woody Plant Basal Media

NAA : a- naphthaleneacetic acid

PGR : plant growth regulator

2,4 : Dichlorophenoxyacetic acid,

2,4-D : silver nitrate,

AgNO3 Indole-3-acetic acid, IAA.

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CHAPTER 1

INTRODUCTION

Black mulberry, known as *M. nigra*, is a member of the *Moraceae* family that has been domesticated over thousands of years and can be grown in different climatic conditions ranging from tropical to temperate. It's believed *M. nigra* is originated from Anatolia, Iran, the Caucasus, and the Levant (Kılınçer et al. 2024).

The primary economic significance mulberry holds is in its leaves, which are used as a food source for the silk-producing bug *Bombyx mori L*, whereas *M. nigra* was among the first to be adapted and cultivated by Greek and Romans for its tasteful fruits and the potential use in pharmaceutical as well as cosmetic industries (Datiles and Acevedo 2022).

As the commercially demand of this plant rises it's conventional propagation methods have been labor-intensive and time consuming, often taking several years to produce mature trees (Yadav et al. 1990), for example multiplying through seeds is constrained by the presence of high rate heterozygosity and a long duration of sexual immaturity required for the attainment of fruit production (Duarte et al. 2019), while cutting can only be done particular months with in the year and very small number of *M. nigra* are generated due to poor rooting frequencies (Desai et al. 2018).

Therefore, to overcome these problems, micro-propagation technique has been applied and become the main gate of bulk production of the clones of valuable *M. nigra* cultivar. Many plant species are vegetatively micro-propagated through various in-vitro methods by using organ cultures or somatic embryos (Litwińczuk and Jacek 2020). In this way, not only will it be possible to cultivate a huge number of plants simultaneously, but it will also be possible to preserve clones of plants produced from tiny sections of explants, ensuring uniformity among the plantlets (Desai et al. 2018).

Plant tissue culture refers to a technique where small pieces of plant material are grown in nutrient rich medium under controlled conditions to develop and multiply for specified time period (Solangi 2022). It is commonly used for micropropagation, creating virus-free planting material, plant multiplication (Seçgin et al. 2018), and modification of genes (Kaya et al. 2013). Secondary metabolites such as phenolics (Ozyigit, 2008), Anthocyanins (Marchev et al. 2020), are also created during the process of tissue culture. Micropropagation is the most common technique in tissue culture. This involves the rapid creation of clean stock plants using small plant parts under sterile controlled conditions in a laboratory setting (Seçgin et al. 2018). Micropropagation is also used to multiply planting materials with superior genetic qualities whose seeds are inefficient under natural vegetative propagation (Santos et al. 2010). The recovery of each single cell into a new plant is an exhibition of a plant tissue's totipotency.

The application of tissue culture innovation to clonal proliferation of alluring characteristics of woody tree species offers reliable alternatives to conventional vegetative propagation (Thorpe 1983). In vitro, culture of tissues, cells, and organs provides unique opportunities for tree improvement (Karnosky 1981). A detailed observation of callus formation from fresh leaf ex-plants of *M. nigra* is hereby presented in this report, though my study intended to develop shoots from the *M. nigra* leaf explants.

CHAPTER 2

LITERATURE REVIEW

2.1. Mulberry Leaves

The leaves of *M. nigra* are essential in sericulture, contributing significantly to the global silk industry. These adaptable plants are prized for their leaves, which are the primary source of nutrition for silkworms during the critical stages of silk production (Chen et al. 2022). Despite rising demand for silk in the biomedical and textile industries, a lack of mulberry trees and short harvesting seasons for mulberry leaves have hampered the development of modern silkworm-rearing farms and weakened sericulture viability (Dong et al. 2017).

M. nigra leaves are also vital in animal nutrition. *M. nigra* leaves have a high protein content, carbohydrates, minerals, excellent amino acid profile, fibers, fats, and metabolizable energy (Hassan et al. 2020). They also have vitamins B1, D, carotene, folic, and folinic acid. However, Copper, zinc, boron, and manganese are in trace amounts (Munțiu et al. 2022). Additionally, *M. nigra* leaves supplement concentrates in dairy cattle, as the primary feed for sheep and goats, and as an ingredient in rabbit and pig diets (Munțiu et al. 2022). Furthermore, *M. nigra* leaves contain more protein and energy than other shrub leaves and traditional forages, and its flavonoids improve milk production, feed digestibility, modulate the chemical processes in the body and reduce harmful free radical damage in animals (Hassan et al. 2020). *M. nigra*, therefore, provide multiple nutritional benefits when compared to conventional forages. *M. nigra* leaves are also medicinal.

M. nigra leaves contain unique components that treat various ailments. The plant's leaves are high in secondary metabolites such as anthocyanins, flavonoids, and alkaloids, which have numerous health benefits such as anti-diabetic, anticancer, antioxidant, and immune-

modulating properties (Qayoom et al. 2023). Also, *M. nigra* leaves are an anti-hyperglycemic nutraceutical for diabetes patients in Korea and Japan (Sarkhel et al. 2020). The primary bioactive compounds found in *M. nigra* leaves include phenols, 1-DNJ alkaloids, and flavonoids, all of which possess significant nutritional benefits and have antibacterial, glucose metabolism, hypoglycemic, lipid metabolism regulation, and anti-inflammatory properties (Yang et al. 2023). *M. nigra* leaves aid in the prevention of a variety of diseases in humans. These advantages have grown in importance over time. *M. nigra* leaves also have non-food properties.

M. nigra leaves are helpful in a variety of non-food industries. The silkworm pupae oil produced by the leaves fed to the silkworm is a critical raw material in making pharmaceuticals, soaps, candles, plastics, paints, varnishes, and biofuels (Jaiswal et al. 2021). Even in the cosmetics industry, the *M. nigra* leaf is used as a raw material (Sarkhel et al. 2020). Various parts of Morus species leaves demonstrate active anti-tyrosinase ability, portraying it as an attractive candidate for the cosmetic industry as a whitener and a key component in dermatological creams, bath gels, and other products (Jan et al. 2021). *M. nigra* leaves have proven their versatility through their ability to be processed into multiple non-food products. Apart from the benefits mentioned above, *M. nigra* leaves purify the atmosphere.

M. nigra leaves purify the air and preserve the environment. Its leaves adapt to harsh climatic conditions, and their high absorption capacity aids in regulating global warming and atmospheric pollution (Rahman and Islam 2021). *M. nigra* leaves help in air purification, thus making the world habitable. *M. nigra* plantations, therefore, improve air quality. *M. nigra* leaves are of great economic value to thousands of farmers who rely on them for income generation.

M. nigra leaves create employment for people. *M. nigra* leaves nourish and rear silkworms, which produce silk yarn. Worms convert *M. nigra* leaf protein into silk protein (sericin and fibroin), which makes high-quality silk garments (Ghosh et al., 2017). *M. nigra* silk accounts for roughly 90% of global raw silk production, significantly contributing to the livelihoods of many people worldwide (Ghosh et al. 2017). Aside from feeding silkworms, the *M. nigra* leaf provides a living for millions worldwide. Thus, Sustainable *M. nigra* production is vital in climate change and variability. As a result, a

standardized method for culturing and regenerating *M. nigra* plant tissues outside their natural environment is required to sustain sericulture.

2.1.1. A universal protocol for efficient in-vitro regeneration of *M. nigra* leaf explants

The science of micropropagation involves the extraction of small plant tissues from a parent stock, and its multiplication under sterile controlled conditions to produce new plants or plantlets (Khan, et al. 2015). Consequently, rapid multiplication of true-to-type disease-free uniform plants occurs since the process is independent of seasonal and weather changes. Due to their availability and ease of manipulation, plant organs such as shoot tips, ovaries, anthers, tendrils, zygotic embryos, and leaves are typically grown in the laboratory setting (Khan et al. 2015). Transgenesis has a high potential for direct organogenesis from explants, particularly cotyledons and leaf segments (Chanotra 2019).

M. nigra in vitro regeneration requires careful collection and preparation of leaf explants. Propagation explants should be disease-free, young, and healthy (Sheena and Jothi 2015). Appropriate *M. nigra* leaf explant selection is critical because *M. nigra* breeding's primary goal is to improve leaf productivity, which accounts for over 38.2% of sericulture productivity (Chanotra 2019). Furthermore, proper explant selection and preparation ensures gene uniformity between propagated crops and mother plants, which leads to higher yields (Gautam et al. 2021). Leaf explants should be collected from mother plants two to three times before they are rejuvenated through sub-culturing for greater precision and accuracy (Segatto et al. 2022). The sterilization of explants is the next step.

In the second protocol, *M. nigra* explants should undergo sterilization to remove contaminants. Microorganisms such as yeasts, molds, fungi, and bacteria pose a significant barrier to a successful micropropagation protocol (Abdalla et al. 2022). Following the presence of contaminants, sterilization is critical in eliminating surface microbes (Rohela et al. 2018). Explants are sterilized with chemical agents such as ultraviolet (UV) sterilization, liquid detergents, antiseptics, mercuric chloride, or sodium hypochlorite, autoclaving of instruments and media, and improvements in cultural practices or handling; antibiotics act as anti-microbial agents in the elimination of in in-vitro cultured plants' endophytic bacteria (Abdalla et al. 2022). Additionally, surface sterilization with running water can prevent external contamination by epiphytic microbes (Abdalla et al. 2022). Explant sterilization is thus unavoidable for researchers attempting

to propagate explants successfully. After sterilization, explants should be transferred to an adequate amount of culture medium.

Explant regeneration requires an adequate and appropriate culture medium. Plant tissue culture media should include some or all of the following elements: vitamins, macronutrients, amino acids, nitrogen and micronutrients, additives, carbon sources, solidifying agents, unspecified organ supplements, and growth hormones. In addition to C, H, and O, tissue culture or plant cell media require macro elements such as magnesium (Mg), nitrogen (N), phosphorus (P), calcium (Ca), potassium (K), and sulfur (S) to ensure proper growth and morphogenesis. Additionally, satisfactory plant cell growth requires a culture media of about 25-60 mM of inorganic nitrogen (Rinaldi et al. 2012).

Plant tissue culture employs several media. One medium widely used in varying concentrations depending on the propagation context is the Murashige and Skoog (MS) Medium (Taha et al. 2020). Murashige and Skoog (MS) medium is regarded as a universal medium for a wide range of herbaceous plants because of its high ion concentration compared to other media, particularly nitrogen, potassium, zinc, and chlorine (Kumara et al. 2022). MS also contains all vitamins, micro and macronutrients, and amino acids required during propagation (Sheena and Jothi 2015). Nitsch and Nitsch (NN), Gamborg (B5), and Linsmaier and Skoog (LS) medium are additional primary media used in tissue culture propagation (Rinaldi et al. 2012). Therefore, the media used in plant propagation differ depending on the explants' specific nutrient requirements and the propagation context. White's media is also used in mulberry propagation.

Researchers have unsuccessfully attempted to use White's media in crop propagation. However, MS and LS media have been mainly used in tissue culture propagation and have often proved better than White's (Ohyama and Oka 1987). These findings corroborate with Qayoom (2023), who discovered that White's media was used in another culture and was examined using three media, including MS media at both original strength and half the concentration as well as white medium. However, only the full-strength MS was efficacious. Therefore, based on previous experiments, White's media is not the best medium for *M. nigra* tissue culture. *M. nigra* explants such as leaf discs have also been propagated in the laboratory under controlled physicochemical conditions,

using axillary buds to produce a whole plant cultured in Gamborg's B5 medium (Sil 2021).

Explants are also developed using Driver and Kuniyuki (DKW) and Woody Plant Medium (WPM) medium (Mirzayeva 2023). WPM (Woody Plant Medium) is the most commonly used medium for the propagation of trees and shrubs. It varies from the most widespread MS (Murashige and Skoog) by having a higher sulphur concentration and a lower nitrogen content (Kumara et al. 2022). Furthermore, in most woody plants, more thinned media, such as Woody Plant Medium (WPM), which contain reduced levels of potassium, nitrogen, and salts, are typically used (Kumara et al. 2022).

Plant growth regulator hormone treatments are vital for efficient in-vitro regeneration of *M. nigra* leaf explants. Plant Growth Regulators (PGRs) are synthetic or natural compounds that, at low doses, affect higher plants' developmental or metabolic processes. In most cases, PGRs disrupt the plant hormone balance in treated plants (Rademacher 2015). It is evident that for effective growth and development, plant growth regulators such as cytokinins and auxins are very crucial. The interaction between these two regulates several development processes notably the formation and maintenance of meristems. Meristems are essential for the establishment of the entire plant structure (Saha et al. 2016).

M. nigra can be grown in soil types and topographical conditions inhospitable to most agricultural crops. Conversely, *M. nigra* usually develop when exposed to temperatures ranging from 13 °C to 37 °C. Still, the maximum sprout of buds occurs when the environmental temperature ranges from 24 °C to 28 °, with a relative humidity of 65-80% (Baciu et al. 2023). Additionally, a light intensity of 3000 lux for a 16-hour photoperiod is required (Rao et al. 2007). In addition to having the cultures under photoperiods of 16 hours during the day, they require 8 hours of night darkness or a supply of 3000 lux fluorescent cool white lamps (El-Homosany and Farag 2020). Also, in in-vitro cultures, the pH of the medium should be in a range of 5.6 to 6.2 (Ilczuk and Jacygrad 2016).

After propagation, the generated crops should gradually be introduced to their new environments. The main goal and critical process in the protocol of in vitro propagation of mulberry varieties is acclimatization and hardening (Taha et al. 2020). Plantlets are typically placed in a greenhouse with planting substrate at high humidity, and after three

weeks of acclimatization, they are usually ready for field transplantation (Cui et al. 2020). At the acclimatization stage, well-rooted plantlets are washed with purified water to eliminate any remaining culture medium and then transplanted (Attia et al. 2014).

2.1.2. Effects of different phytohormones and plant growth regulators on *M. nigra* *In vitro* regeneration

İn vitro regeneration of *M. nigra* involves using phytohormones and plant growth regulators. Plant hormones, also known as phytohormones, are naturally occurring substances produced internally (endogenously) within plant tissues and are responsible for enhancing vegetative growth (Singh et al. 2021). On the other hand, plant growth regulators are synthetic chemical compounds with physiological effects on plant growth substances and can alter vegetative growth (Singh et al. 2021). The main plant growth regulators include auxins, abscisic acid, ethylene, gibberellins, and cytokinins (Singh et al. 2021).

Auxins are hormones that stimulate plant growth and the development of leaf, fruit, shoot, and lateral roots (Qayoom et al. 2023). Auxins also help to mobilize carbohydrates from the leaves and upper stem, and accelerate their distribution to the rooting zone (Husen et al. 2017). Additionally, auxins improve histological features such as callus, tissue formation, and vascular tissue differentiation (Singh et al., 2014). Indole-3-butyric acid (IBA) and Indole-3-acetic acid (IAA) INAA are the most recommended auxins in regeneration because they promote tree and shrub propagation by causing metabolic changes during adventitious root formation (Husen et al. 2017; Sokhuma et al. 2018). Root formation occurs through three distinctive yet sequential phases i.e. induction, initiation, and expression. Although no visible changes occur during the induction phase, underlying molecular and biochemical processes are ongoing. The initiation phase is characterized by cell division and formation of root primordia which grow internally and eventually emerge during the emergence stage (Husen et al. 2017).

Cytokinins help increase the number of shoots produced, especially at higher concentrations (Karyanti et al. 2021). Cytokinins, particularly 6-Benzylaminopurine (BAP), are effective for shoot induction and proliferation in mulberry cultures (Qayoom et al. 2023) and are also effective in shoot multiplication (Taha et al. 2020b). Kinetin is another plant proliferation cytokinin; however, most of its explants exhibit inferior

responses when added to the culture medium; thus, Kinetin is considered one of the least effective cytokinins (Radi et al. 2013).

In most instances,cytokinin BAP has proven more effective than Kinetin in axillary shoot proliferation (Rana et al. 2022). However, some researchers have established that for Kinetin to be effective in shoot multiplication, it must be supplemented with other cytokinins, such as BA (Karyanti et al. 2021). Gibberellic acid (GA3) is also infrequently used to improve mulberry shoot elongation and overall growth, under low moisture conditions, GA3 at 0.05 to 1.0 mg/l in combination with BAP promotes shoot elongation and senescence but inhibits leaf enlargement (Karyanti et al. 2021).

Table 2.1. Overview of the functions of different plant growth regulators in tissue culture

Plant growth regulator	**Role in tissue culture/ *in vitro* propagation**		
		Natural	**Synthetic**
Auxin	• Callus and suspension cultures initiation and proliferation • Induce somatic embryos • Adventitious shoot formation in selected species	IAA	IBA NAA; 2,4-D 2,4,5-T
Cytokinin	• Formation of adventitious shoots • Overcome apical dominance, thus allowing shoot bud proliferation • When combined with auxins, it causes explant cell division • Induce somatic embryos	Zeatin BA	Kinetin TDZ
Gibberellins	• Shoot elongation	GA1 GA3 GA7	
Abscisic acid	• Somatic embryos maturation • Promote shoot budding and somatic embryo regeneration	ABA	
Polyamine	• Promote shoot formation	Spermidine Putrescine Spermine	
Source: (Lin et al., 2021; Sil, 2021; Qayoom et al., 2023)			

Gibberellins (GAs) are carboxylic acids regulating seed germination, leaf growth, flower development, stem elongation, and trichome initiation (Chen et al. 2022). Conversely, salicylic acid (SA) is a natural molecule that regulates plants' resistance to environmental stresses such as drought, low temperatures, salinity and osmotic pressure, and heavy metals, and also induces thermotolerance (Khan et al. 2019; Peng et al. 2015). SA also promotes various biochemical and physiological processes, such as ion uptake, photosynthesis, stomatal opening, flower induction enzymatic activity, and membrane permeability (Mugwanya et al. 2023). In addition to the above effects, SA helps regulate the expression of pathogen-related proteins like viruses, bacteria, and fungi, thereby protecting plants from diseases (Chen et al. 2022).

Aside from salicylic acid, Abscisic Acid (ABA) also aids plants in dealing with environmental stress (Li et al. 2023). ABA influences root development, seed germination, and the plant's reaction to stress by stimulating the production of ethylene, and ABA's interaction with auxins and ethylene regulates abiotic stress tolerance (Zhu et al. 2023). Furthermore, ABA regulates somatic embryo development by binding to specific receptors in the plasmalemma, nucleus, and chloroplast (Phillips and Garda 2019).

ABA's role in regulating the maturation of embryos results in the formation of high-quality somatic embryos that can tolerate desiccation (Zein et al. 2022). However, low levels of ABA are needed to produce and elongate adventitious roots (Sun et al. 2023). However, ethylene is a good regulator of plant growth and development due to its genes involved in ethylene gesticulating and biogenesis are significant targets for improving leaf and fruit yield (Shang et al. 2014).

2.1.3. Factors affecting the ability of *M. nigra* leaves explants to produce adventitious shoots

M. nigra in vitro propagation is dependent on genotypes and explants and uses various plant parts, including leaves (Sakar et al. 2023). *M. nigra* leaves explants produce adventitious roots based on the explant's age and genotype; explants from young leaves regenerate more frequently than old ones (Vijayan and Tikader, 2014).

The ability to produce adventitious shoots is dependent on the composition of the nutrient medium, such as cytokinins or plant growth regulators, which are plant growth hormones that stimulate shoot initiation and growth in many plants (Larekeng et al. 2021). Plant growth regulators influence shoot multiplication, and their effect depends on the type and concentration of cytokinins and auxins (Rezaei and Rahmati 2020). Cytokinins promote shoot initiation by stimulating cell or tissue division, which supports shoot development. Furthermore, cytokinin breaks dormancy and promotes buds' lateral branching (Larekeng et al. 2021).

Benzyl adenine (BA), also called 6-benzyl aminopurine, is the most common plant growth regulator for initiating cell division and shoot differentiation in plant tissue culture (Mangena 2020). Another synthetic cytokinin that increases shoot formation from mulberry leaf explants is Thidiazuron (TDZ). TZD has proven to generate extra shoots per explant than BAP at the same concentration and with explants of the same age; TDZ is thus effective in shoot induction in mulberry (Thomas 2003). Moreover, TDZ improves regeneration efficiency and induces shoot induction without the formation of calluses (Sajeevan et al. 2011).

When combined with cytokinins, auxins promote shoot formation in plant tissue culture. The balance of cytokinins and auxins is critical for meristem growth and the formation of new shoots (Grzegorczyk et al. 2021). Indole-3-acetic acid (IAA) can be utilized singly or combined with other plant growth regulators to provide the best results (Sil 2021). For instance, incorporating IAA into a TDZ-containing medium improves shoot regeneration (Guo et al. 2011). However, a high cytokinin-to-auxin ratio is required to regulate cell division and shoot morphogenesis (Larekeng et al. 2021).

Another factor is pH. Maintaining an appropriate pH level in the culture medium (usually around 5.8) is critical for shoot regeneration (Abdalla et al. 2022b). Apart from the pH, proper sterilization of explants is crucial because microorganisms often thrive fast in tissue culture. Some of the surface disinfectors include antibiotics, fungicides, sodium hypochlorite (NaOCl), calcium hypochlorite (CaOCl), ethanol (EtOH), silver nitrate (AgNO3), hydrogen peroxide (H2O2), and mercuric chloride (HgCl2) (Parzymies 2021). Due to the potential phytotoxicity of disinfectants, obtaining pathogen-free plant tissue

cultures can be difficult. To avoid plant tissue damage and necrosis, disinfectant type, concentration, and exposure time must be carefully controlled (Parzymies 2021).

Lighting is also crucial in the formation of adventitious shoots from explants. For most propagation processes, it is recommended to use a 16-hour light and 8-hour dark cycle with a light intensity of 3,000 lux from cool white fluorescent lights and maintain the growth room temperatures at 26^0C (Attia et al. 2014). Growing mulberry varieties and hybrids in vitro requires a room temperature of 20- 220 degrees Celsius and a humidity of approximately 60 to 70 percent (Mirzayeva 2023b)

2.1.4. Morphological and physiological changes through which the *M. nigra* leaves explants undergo to produce adventitious shoots

Explants go through multiple changes to produce adventitious shoots. The first stage or change in most in vitro regeneration is callus formation, which occurs when differentiated cells dedifferentiate to become pluripotent (Wang et al. 2022). The formation of calluses is thus the first step in tissue culture and serves as a source of cells for subsequent shoot initiation. Another change that occurs is embryogenesis or somatic regeneration, an induced process where a plant embryo develops from an individual somatic cell. Cell division commences immediately after leaf explants are cultured on a regeneration medium, but only a limited number of these cells will differentiate into shoot meristems (Subban et al. 2020). However, these primordia are not discernible during the first days (Subban et al. 2020). Small clusters or groups of cells begin to differentiate and form structures known as shoot primordia within the callus tissue, which are the early stages of shoot development (Subban et al. 2020). The meristemoid formation is another change that occurs during in vitro propagation.

Meristemoid formation before adventitious buds emerge is a typical histologic process in initiating new shoot development. This formation suggests that the underlying molecular processes involved in organogenesis are consistent across different plant explants, emphasizing their similarities (Da Silva et al. 2020). Organogenesis is a complex phenomenon that involves the formation of organs from scratch. Plants develop directly from explants in direct organogenesis, whereas plants develop indirectly through callus formation in indirect organogenesis (Chanotra 2019).

During organogenesis, shoot primordia emerge as visible shoots from the callus tissue, which may initially be small and fragile (Thomas 2002). As the shoots develop leaves, the overall morphology of the plantlets resembles that of mature mulberry plants; this is followed by root formation, which may form from the base of the shoot plantlets depending on the tissue culture conditions and the specific objectives (Thomas 2002). The induction of adventitious shoot formation in mulberry leaf explants frequently necessitates plant growth regulators or hormones where cytokinins and auxins are the main hormones involved (Balakrishnan et al. 2009). Varying concentrations of auxins and cytokinins affect callus formation in in-vitro propagation (Balakrishnan et al. 2009). High IAA concentrations promote root meristem cell division, whereas low concentrations promote root elongation zone cell differentiation (Sun et al. 2023).

CHAPTER 3

MATERIALS AND METHODS

3.1. Plant materials

The seeds of *M. nigra* were obtained from Kayseri in Talas, Turkey. The mature seeds were germinated on the soil after soaking it in 1grGA in one litter water concentration for three days at room temperature. This enables a germination percentage of up to 83% within 14 -20 days. Two types (cotyledon and mature fresh leaves) were collected from the healthy seedlings and used in this experiment

A B

Figure 3.1. (A) and (B) show the cotyledon and fresh young leaf explants extracted from *M. nigra* that were used in carrying out the study.

3.2. Surface sterilization

The leaf explants were gathered and rinsed in running water for 20 minutes. After this initial washing, the explants were sterilized for 5 minutes in a 1% (w/v) fungicide solution (Ancom Thiram, USA). Subsequently, the explants were rinsed twice with sterilized distilled water with each rinse lasting between 3 to 4 minutes. Sterilization of the explants was again conducted in 0.1% $HgCl_2$ (Mercury chloride) for 5 minutes. This

was followed by 3 - 4minute sterilization using distilled water to rinse the explants in four rounds/times.

3.3. Culture media

The study utilized three different basal media formulations as foundations for the plant tissue culture experiment. Standard MS (Murashige and Skoog 1962) medium, Lloyd & McCown Woody Plant Basal Medium, and Blue Berry medium were used as a solid media. To evaluate the optimal concentration of media for inducing callus on *M nigra*, full and half-strength (½) MS, WPM and blueberry media were formulated. These were enhanced with a range of PGRs at varying concentrations. A control was set up as a medium lacking any PGR. In preparing MS, WPM, and blueberry media, micronutrients, macronutrients, vitamins, and FeNaEDTA were included and the medium was enriched with 3% (w/v) sucrose. The plant growth regulators were added prior to autoclaving. The pH was set to 5.7 ± using 1M NaOH and solidified with 0.8% (w/v) agar. The media were then autoclaved at 121°C for 15 min. Approximately 25 mL of the sterile culture medium was then poured into a sterile disposable petri dish measuring 100 mm × 15 mm each (Labchem, Malaysia).

A B C D

Figure 3.2. Show the culture establishment of *M. nigra* leaf explants in various tissue culture medium with varying PGRs after sterilization. Explants in blueberry media (A and B), WPM media (C), and MS media (D).

3.4. Callus initiation

The surface sterilized leaflets were cultured on either half or full-strength MS, WPM, and blueberry solid media. These media were supplemented with various PGRs including auxins including like Indole-3-acetic acid (IAA), 2,4-Dichlorophenoxyacetic acid (2,4-D), a-naphthaleneacetic acid (NAA)] and cytokinin including like Zeatin, Thidiazuron (TDZ), 6-benzyl amino purine (BAP), and silver nitrate at concentrations of 0.0, 2.5, 5.0, 7.5, 10.0, and 20.0 μM. Each petri dish contained 5 explants for each treatment with three replicates performed and repeated three times.

3.5. Culture conditions

The sterilized leaf explants were placed by their abaxial sides on the growth medium and with the upper surfaces oriented upwards. Explants were kept in the dark for 2-3 weeks in a thermostat MIR-254 (SANYO, Japan) at a temperature of 24±1 °C. After this period, they were transferred to a growth chamber MLR-352-PE (SANYO, Japan), maintained at the same temperature, and subjected to a 16-hour light cycle using cool white fluorescent lamps (Philips TL, 40W) at a light intensity of 37.5 μmol m-2 s-1. Every 4 weeks, the explants were subcultured. Observation of the percentage of callus induction, duration of formation, morphology and colour, growth intensity, and root formation was conducted every 5 days.

3.6. Statistics analysis

The data collected in this study was subjected to statistical analysis. One-way analysis of variance (ANOVA) and Tukeys post-hoc test known as the Honestly Significant Difference (HSD) tests, were used to compare the mean values of measured parameters at a significant level of $p<0.05$. The statistical software employed in the analysis was SPSS version 11.5 (SPSS Inc. USA).

CHAPTER 4

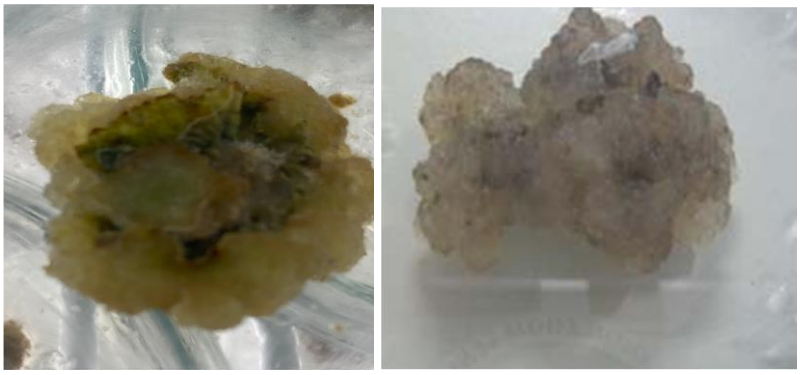
RESULTS

4.1. Effects of medium strength on callus induction

Various media yielded distinct effects on callus induction (Tables 1, 2, 3, and 4). The results were evaluated in terms of the day of callus induction, the intensity of callus growth, and the morphology of the callus formed.

4.1.1. Standard MS (Murashige and Skoog 1962) solid media

Within the four weeks of culture, all MS medium supplemented with TDZ (2.5, 5.0, 7.5, 10.0, and 20.0 µM) effectively induced callus formation from leaf explants of *M. nigra*. The fasted callus formation was observed in 15 days in explants cultured in ½ MS medium supplemented with 7.5 µM TDZ which resulted in abundant white yellowish callus (fig. 3 A). Conversely, the slowest callus formation was observed in 19 days in explants cultured in full MS medium supplemented with 7.5 µM TDZ. This was associated with unhealthy white-brownish callus (fig. 3 B).



A B

Figure 4.1. Callus induction from the leaf explant of *M. nigra* on ½ MS medium supplemented with 7.5 µM of TDZ that formed yellowish callus (A) while in full MS media supplemented with the same TDZ concentration that formed White Brownish callus (B).

The results further indicate that the strength of the MS medium significantly influenced callus induction from leaf explants of *M. nigra*. Varying results in terms of callus morphology and duration of callus initiation were observed in explants supplemented with different strengths of MS medium but same concentration of TDZ (7.5 µM). The ½ MS medium was observed as the most effective for callus induction. This observation aligns with findings in *Taxus* species where successful induction and poor callusing were observed in on ½ MS media and full MS media respectively (Chang and Yang 1995).

Table 4.1. Effect of full- and ½ MS medium supplemented with TDZ at various concentrations on the callus induction and characteristics of callus derived from the leaf explant of *M. nigra*

MS Media Strength	**TDZ concentration (µM)**	**Days of Callus**	**Degree of Callus**	**Miorphology of Callus**	**Colour of Callus**	**Root initiation**
½ MS	0	-	-	-	-	Negative
	2.5	20	+	Compact	WB	Negative
	5.0	17	++	Friable	WB	Negative
	7.5	15	+++	Friable	WY	Negative
	10	16	++		WY	Negative
	20	27	+	Compact	WY	Negative
MS	0	-	-	-	-	Negative
	2.5	25	+	Friable	WB	Negative
	5.0	22	++	Compact	WY	Negative
	7.5	19	++	Friable	WB	Negative
	10	28	+	Compact	WY	Negative

Callus growth scoring value = +: Poor, + +: Moderate, + + +: Profuse

Colour of callus formed = W: White, WY: Whitish Yellow, WB: Whitish Brown; GW, Greenish watery.

Root formation rating = Positive: Roots formation, Negative: No root formation

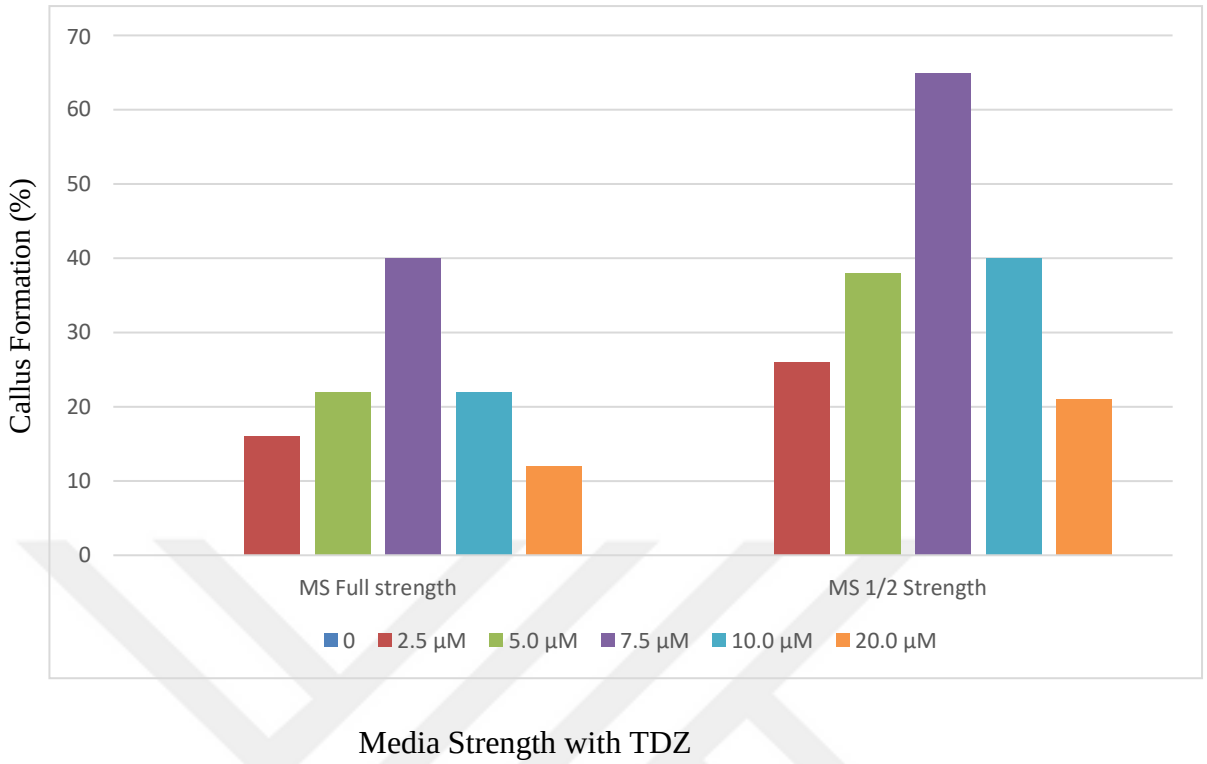


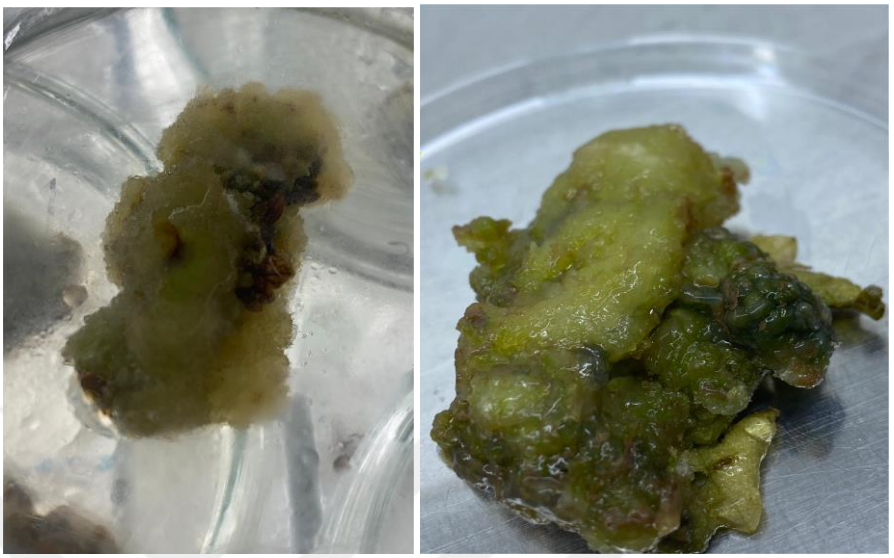
Figure 4.2. Percentage of callus induction from the leaf explants of *M. nigra* after 4 weeks of culture on ½ and full MS media supplemented with various concentrations of TDZ. Bar indicates the mean ± standard deviation.

4.1.2. Lloyd & McCown Woody Plant Basal Medium

Within 5 weeks of culture, successful callus induction from leaf explants of *M. nigra* was observed in all the WPM medium supplemented with TDZ (2.5, 5.0, 7.5, 10.0, and 20.0 µM). The shortest callus formation time of 12 days of culture was exhibited in explants cultured in full WPM medium supplemented with 10 µM TDZ. Abundant growth of friable and whitish callus (fig. 5 A) was observed in this treatment. The longest callus formation time of 19 days of culture was observed in explants cultured in ½WPM medium supplemented with 10 µM TDZ characterized by greenish watery callus.

The strength of the WPM medium significantly affected callus induction from the leaf explants of *M. nigra*. A variance in the morphology of callus and callus initiation was observed in explants exposed to different strengths of WPM medium with the same component of concentration of TDZ (10 µM). Thus, this is an indication that full strength WPM medium was more effective for callus induction. This finding is consistent with observations in *Bougainvillea species* where full WMP medium successfully induced

callus induction and half strength WPM media exhibited poor callusing (Takashina, et al. 2002).



A B

Figure 4.3. Callus induction from the leaf explant of *M. nigra* on full strength WPM medium supplemented with 10 µM of TDZ that formed white yellowish callus (A) while in ½ WPM media supplemented with the same TDZ concentration that formed Green watery callus(B)

Table 4.2. Effect of full- and ½ WPM medium supplemented with TDZ at various concentrations on the callus induction and characteristics of callus derived from the leaf explant of *M. nigra*

WPM Media Strength	**TDZ concentration (µM)**	**Days of Callus initiation ***	**Degree of Callus formation**	**Morphology of Callus**	**Colour of Callus**	**Root formation**
WPM	0.0	-	-	-	-	Negative
	2.5	22	+	Friable	WB	Positive
	5.0	20	++	Friable	WY	Positive
	7.5	18	++	Watery	WY	Negative
	10.0	12	+++	Watery	WY	Negative
	20.0	16	+	Compact	WY	Negative
½ WPM	0.0	-	-	-	-	Negative
	2.5	36	+	Friable	WB	Positive
	5.0	30	+	Watery	WY	Positive
	7.5	26	+	Watery	GW	Positive
	10.0	19	+	Watery	GW	Negative
	20.0	23	+	Compact	WB	Negative

Callus growth scoring value = +: Poor, + +: Moderate, + + +: Profuse

Colour of callus formed = W: White, WY: Whitish Yellow, WB: Whitish Brown; GW, Greenish watery,

Root formation rating = Positive: Roots formation, Negative: No root formation

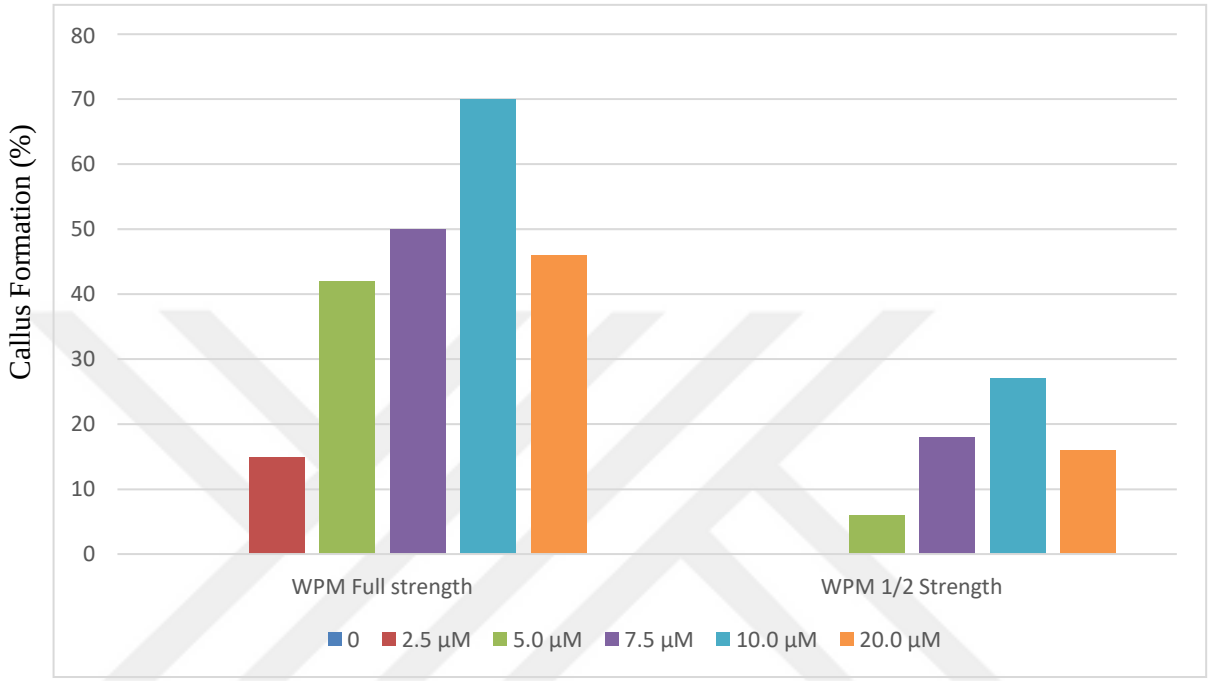
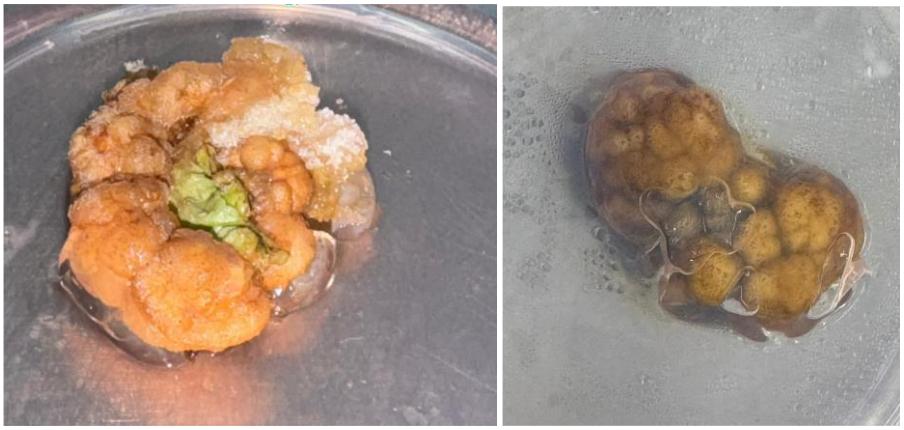


Figure 4.4. Percentage of callus induction from the leaf explants of *M. nigra* after 5 weeks of culture on ½ and full WPM media supplemented with various concentrations of TDZ.

4.1.3. Blueberry medium

Within 4 weeks of culture, it was also observed that only Blueberry medium (**a discovery yet to be published**) supplemented with zeatin at concentrations of 5.0, 7.5, 10.0, and 20.0 µM successfully induced the callus from the leaf explants of *M. nigra*. The shortest callus formation time of 9 days of culture was observed in full Blueberry medium supplemented with 20µM zeatin. Abundant growth of white-yellowish callus was observed in this treatment (fig. 7 A and B). On the other hand, ½ Blueberry didn't produce any callus after 5 weeks.



A B

Figure 4.5. Callus induction from the leaf explant of *M. nigra* on full strength blueberry medium supplemented with 20 µM of TDZ that formed white yellowish callus (A) while in ½ blueberry media supplemented with the same Zeatin concentration that formed White yellowish callus (B).

Table 4.3. Effect of full- and ½ Blueberry medium supplemented with Zeatin at various concentrations on the callus induction and characteristics of callus derived from the leaf explant of *M. nigra*

Blueberry Media Strength	**Zeatin concentration (µM)**	**Days of callus intiation ***	**Degree of callus Formation**	**Morphology of Callus**	**Colour of Callus**
½ Blueberry Media	0.0	-	-	-	-
	2.5	-	-	-	-
	5.0	-	-	-	-
	7.5	-	+	-	-
	10.0	15	+	Compact	WY
	20.0	18	++	Compact	WY
Blueberry media	0.0	-	-	-	-
	2.5	-	++	-	-
	5.0	22	++	Compact	WY
	7.5	16	++	Compact	WY
	10.0	9	+++	Compact	WY
	20.0	14	+++	Compact	WY

Callus growth scoring value = +: Poor, + +: Moderate, + + +: Profuse
Colour of callus formed = W: White, WY: Whitish Yellow, WB: Whitish Brown; GW, Greenish watery.
Root formation rating = Positive: Roots formation, Negative: No root formation.

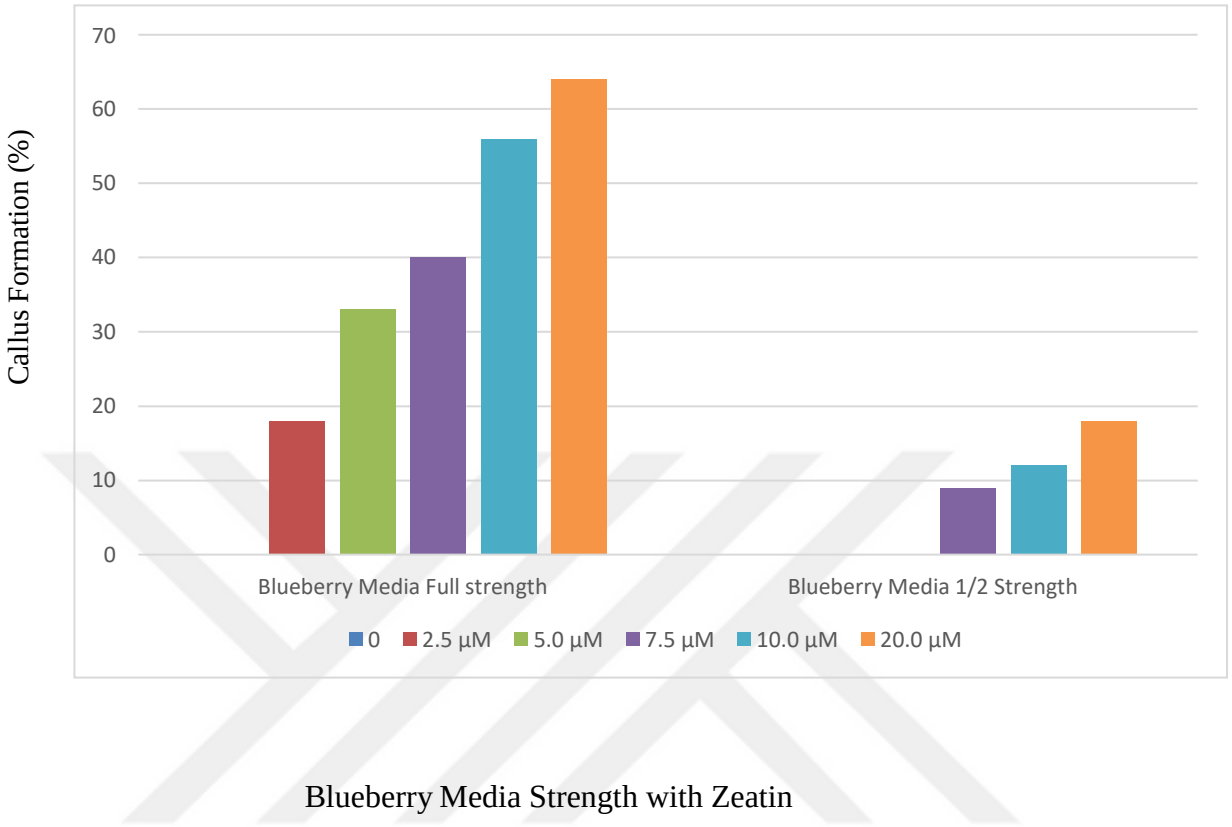
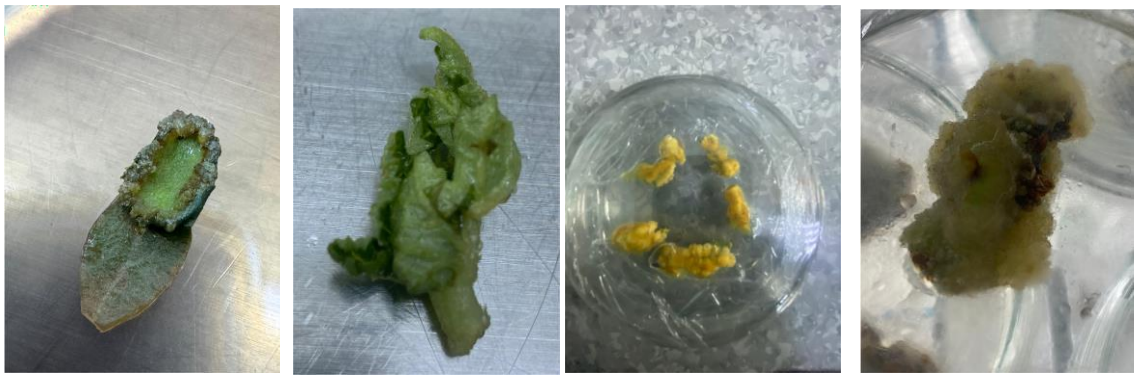


Figure 4.6. Percentage of callus induction from the leaf explants of M. nigra after 4 weeks of culture on ½ and full Blueberry media supplemented with various concentrations of Zeatin.

4.1.4. Effects of plant growth regulators on callus induction with WPM.

The effectiveness of various PGRs in inducing callus has been extensively documented across different plant species, highlighting their essential role in the initiation of callus in vitro (Qi et al. 2005). This study has observed that the concentration of PGRs significantly influences the induction of callus from leaf explants of *M. nigra*. Leaf extracts cultured on PGR-free media (Control) did not produce any callus and eventually turned brown leading to necrosis after 3 weeks of culture. Chne and Chnag (2001) noted that leaf explants of *Oncidium* showed no signs of callus formation when cultured on PGR free medium ultimately turning dark and dying after several weeks.



A B C D

Figure 4.7. Callus induction and morphological changes from the leaf explant of M. nigra in the culture of WPM medium supplemented with various concentrations that formed of PGR (A, B, C, and D).

Table 4.4. Effect of full-strength WPM media supplemented with NAA and 2,4-D at the concentrations of 0.0, 2.5, 5.0, 7.5, 10.0, and 20.0 µM on induction of callus and characteristics of callus derived from the leaf explant of *M. nigra*.

Treatment	**Concentration (µM)**	**Days of callus initiation ***	**Degree of callus formation**	**Morphology of callus**	**Colors of callus**	**Initiation of root**
NAA	0.0	-	-	-	-	Negative
	2.5	9	+	compact	WB	Negative
	5.0	11	+++	Friable	WB	Negative
	7.5	10	+++	Friable	WY	Negative
	10.0	13	++	Compact	WB	Negative
	20.0	16	+	Compact	WY	Negative
2,4 – D	0.0	-	-	-	-	Negative
	2.5	15	+	Friable	WB	Negative
	5.0	13	+++	Friable	W	Negative
	7.5	10	+++	Friable	W	Negative
	10.0	15	++	Friable	WY	Negative
	20.0	19	+	Compact	WB	Negative

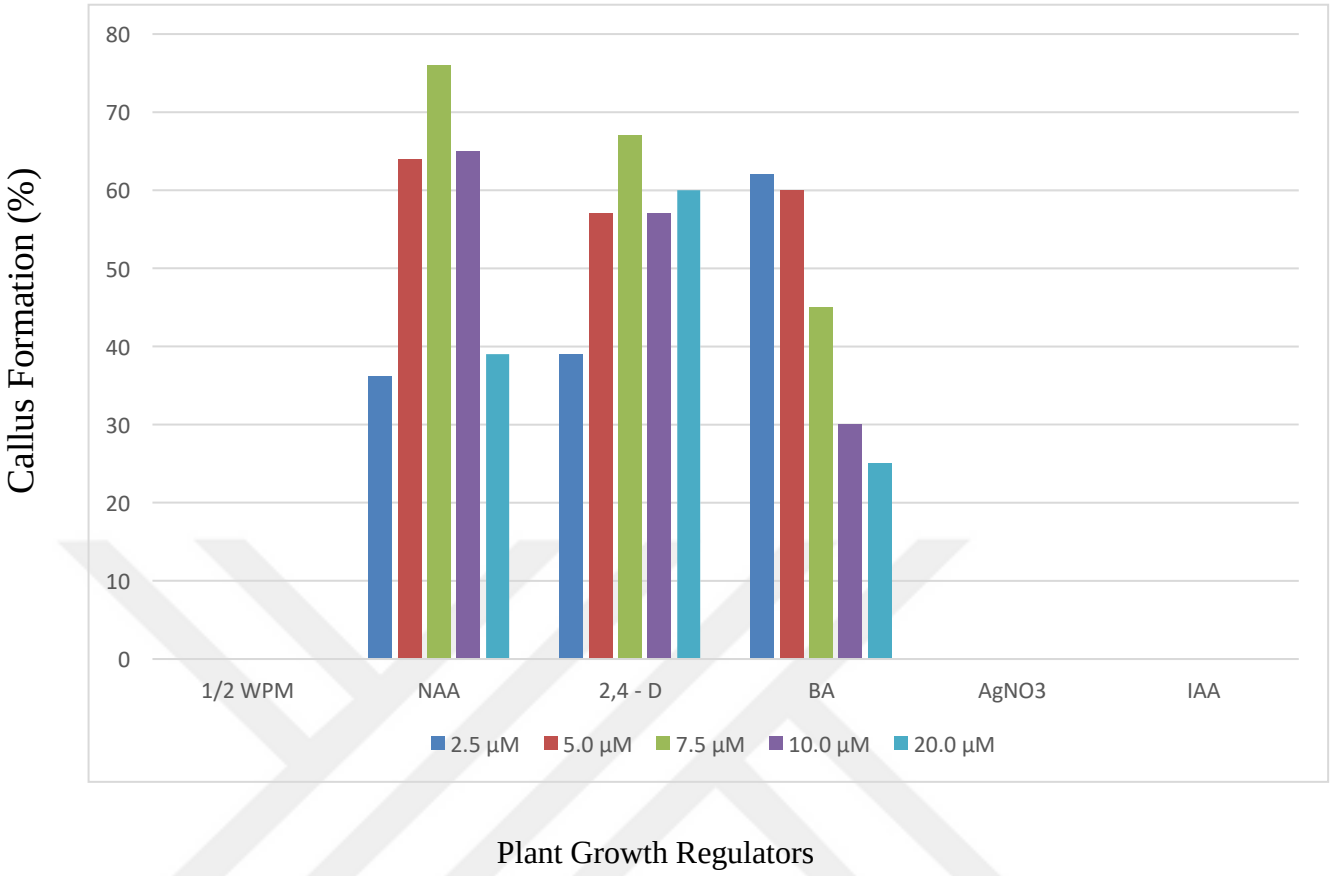


Figure 4.8. Percentage of callus induction from the leaf explants of M. nigra in culture on ½ and full-strength WPM media supplemented with various concentrations of PGRs.

CHAPTER 5

DISCUSSION

According to Islam et al. (2004), callus induction in *Oryza sativa L* was significantly affected by MS medium. When culturing leaf explants of *Brassica* of ½ MS medium, Kester and Hesse (1995) observed the production of friable and whitish callus. They also observed inhibited callus growth when full MS medium was used growth leading to browning and death of the callus after 4 weeks of culture. Given that the concentration of salt is lower in ½ MS medium than full MS medium, it can be hypothesized that high salt concentration may inhibit callus induction from leaf explants *M. nigra*. Further, Jin, et al. (2009) reported that WPM medium strength significantly affected the callus induction in *Zelkova schneideriana*. Additionally, it was reported that WPM medium significantly affected the callus induction in guayule leaf explants (Kang and McMahan, 2014), Northland blueberry (Zhao, et al. 2011), *Bougainvillea sp*. (Duhoky, et al. 2014)

Callus formation at the cut ends was observed to extend gradually across the entire surface of the explants that were treated with NAA. Since different auxins exhibited different levels of callus induction (Table 5), the best callus induction (63%) was observed in 7.5µM NAA. However, within the same treatment, profuse and yellowish healthy callus (fig. 9 D) was observed with callusing taking place after 10 days of culture. This is consistent with findings from Ahmad et al. (2010) who observed whitish callus induction within 16 days in *Ruta graveolens L*. plant.

Lower concentrations of NAA (2.5 µM) were associated with decreased callus induction percentage from 63.0 to 21.2%. After 4 weeks of culture, a greenish watery callus was observed within the same treatment (fig. 5 B). Similarly, lower concentrations of NAA were observed to inhibit callus formation in explants of *Stevia rebaudiana Bertoni* by Blinstrubienė et al (2020). Conversely, retarded somatic embryo formation and cytotoxicity to the cell as a result of high concentration of NAA is reported by (Rashmi

and Trivedi, 2014) on *Nerium odorum* and (Abdelmaksood et al. 2017) on *Hyoscyamus muticus L.*

Synthetic auxin 2,4-D can stimulate cell division of tissues in several plants. This has made it the most widely used auxin to induce callogenesis (Da Silva et al. 2005). In all concentrations tested in this study (2.5, 5.0, 7.5, 10.0, and 20.0 μM), 2,4-D successfully induced callus formation. However, a higher callus induction was observed in NAA treatments (Table 5) compared to 2,4-D.

The highest percentage of induction among the 2,4-D treatments was observed in leaf explants in a WPM medium supplemented with 7.5 μM 2,4-D (Fig. 5 A) with callus formation observed after 10 days of culture. Similarly, optimal callusing responses in legume tissues treated with 5.5 to 7.5 μM of NAA were reported by Chernova et al (2005). Callus induction from the leaf explant of *M. nigra* was negatively impacted by both the lower and higher concentrations of 2,4-D. Reducing concentration from 7.5 to 2.5 μM or increasing it from 7.5 to 20.0μM resulted in reduced callus induction rates from 73.0% to 49.7% and 41.3% respectively (fig. 3 B) with poor, brownish callus observed. A study on the vegetative propagation of *Freesia* through callus cultures by Bajaj and Pierik (1994) who used low concentrations of 2,4-D equally yielded the same results characterized by retarded callus growth. Additionally, a high concentration of 2,4-D was reported to affect the callus texture and morphology in leaf explants of *Nigella sativa* by Banerjee and Gupta (1996).

Lu (2002) noted the rooting of plant species such as *Morus latifolia* and *Morus alba* in the presence of 2,4-D. Although 2,4-D demonstrated a high percentage of callus initiation after 1-week, adventitious roots began to develop from the callus after day 12. Eventually, the growth of callus was hindered and subsequently stopped by the fourth week. This study revealed that root formation was an undesirable occurrence in callus studies as the roots that formed would compete for nutrients and space with the callus thus suppressing its growth (Leroux 1997).

The most commonly known synthetic auxin in facilitating embryogenesis or organogenesis and promoting the development of good quality callus in medicinal plants is IAA (Hu et al. 2011). NAA-supplemented media was reported to have a positive effect on callus growth in *Solanum Melongena L.* (Rahman 2007). Iqbal et al (2021) observed

a successful stimulation of callogenesis in *citrus jambhiri L* as a result of IAA. However, contradictions related to the plant specificity effect of PGRs influencing IAA were noted in the same study because different plant species responded differently. Regardless the concentration tested, IAA failed to stimulate callus formation in leaf explants in *M. nigra*. After the third and the fourth weeks of culture, explants turned brown, and necrosis set in respectively. Oxidation of phenolic compounds which causes browning could be responsible for the occurrence of this phenomenon. I was really not impressed with the quality of calluses produced when i used BA hormone.

The accumulation of phenolics is a reaction to microbial infection or physiological stress which is a part of plant defense mechanism. The production of these phenolic compounds limits microbial invasion into cells (Rahman and Punja 2005). In response to cutting stress within this study, it was observed phenolic compounds were released as a result of the damage caused to the tissues through wounding of leaf explants.

The stress exerted by the detrimental effects of IAA since it has herbicidal properties could be responsible for the observable browning effect (Aslam et al. 2005). The biggest hindrance to proper tissue multiplication and maintenance in plant tissue culture studies over the years has been browning and thus undesirable (Alemanno et al. 2003). While studying *Pinu sylvestris*, phenolic oxidation was reported as the leading cause of browning and eventually necrosis by Anderson and Levinsh (2002)

According to Shah et al (2014), the interaction between AgNO3 and ethylene results in the induction of multiple shoots per plant as evidenced in their study of tomatoes. The addition of ethylene in field-collected male immature inflorescence cultures of buffalo grass significantly increases the induction frequency and growth of embryonic callus (Fei et al. 2020). However, this study produced contradictory results. Since various plants respond differently to PGRs, it can be hypothesized that AgNO3 was affected by the plant-specific nature of PGRs. In the case of *M. nigra*, regardless of the concentration, AgNO3 was ineffective in inducing callus formation from leaf explants. The explants turned brown and became necrotic after the fourth and fifth weeks of culture respectively.

Much as cytokinin is known to promote cell enlargement and division in certain tissues (Ross and Rayle 1982), there were no signs in this study of callus formation after the addition of BAP as a source of cytokinin in the ½ WPM medium. Instead, explants of *M.*

nigra turned yellowish and brownish in the third and fourth week of culture respectively. This is consistent with the findings from Welander (2002) who made the same observations when BAP was used to treat *Begonia hiemalis*. This was further supported by Tanaka and Sakanishi (1998) who report possibilities of callus formation inhibition by higher concentrations of BAP. Low concentrations of BAP in *Haworthia* cultured in MS media did not induce callus (Kaul and Sabhar-wal, 2002).

5.1. Conclusions

Friable calli exhibit significant potential in suspension cell cultures. This calls for further research using in vitro regeneration using leaf-derived callus to enhance the propagation efficiency of *M. nigra*. This could facilitate a higher multiplication rate through vitro propagation compared to traditional techniques. It has been established that callus tissues serve as an alternative source for secondary metabolites of *M. nigra*. Therefore, further research to produce secondary metabolites from leaf-derived callus of *M. nigra* should be undertaken.

5.2. Acknowledgements

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