BOLU ABANT IZZET BAYSAL UNIVERSITY

THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

DEPARTMENT OF BIOLOGY

IN VITRO PROPAGATION OF EINKORN (*TRITICUM MONOCOCCUM* SSP. *MONOCOCCUM*) WHEAT

MASTER OF SCIENCE

MEHMET ÖRGEÇ

BOLU, APRIL 2019

APPROVAL OF THE THESIS

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In vitro **propagation of einkorn (*Triticum monococcum* ssp. *monococcum*) wheat** submitted by **Mehmet ÖRGEÇ** and defended before the below named jury in partial fulfillment of the requirements for the degree of **Master of Science** in **Department of Biology, The Graduate School of Natural and Applied Sciences of Bolu Abant İzzet Baysal University in 30.04.2019** by

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TO MY PARENTS

MUSTAFA MURAT ÖRGEÇ AND GÜLFER ÖRGEÇ

DECLARATION

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Mehmet ÖRGEÇ

ABSTRACT

***IN VITRO* PROPAGATION OF EINKORN (*TRITICUM MONOCOCCUM* SSP. *MONOCOCCUM)* WHEAT**
MSC THESIS
MEHMET ÖRGEÇ
BOLU ABANT IZZET BAYSAL UNIVERSITY GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOLOGY
(SUPERVISOR: PROF. DR. NUSRET ZENCİRCİ)

BOLU, APRIL 2019

Einkorn wheat (*Triticum monococcum* ssp. *monococcum*) has a promising value in food industry, biotechnical approaches and genetic transformation studies as well as agricultural areas due to its desired genetic characters and high nutritional contents. The aim of this study is to produce an efficient tissue culture protocol of einkorn (*Triticum monococcum* spp. *monococcum*) wheat by using different einkorn explant sources with different concentration of some hormones and MS. In the present study, root, leaf, and coleoptile explant sources of einkorn wheat was exploited for callus formation and regeneration. Explants of 5-days *in vitro* germinated seedlings of einkorn were cultured on three different concentration of MS medium (1.1, 2.2, and 4.4 g/L) supplemented with ten different concentrations of 2,4-D (0, 0.5, 1, 2, 3, 4, 5, 6, 8, and 10 mg/L) for callus induction. Callus were then cultivated on six different concentrations of TDZ (0, 0.5, 1, 2, 3, and 5 mg/L) with 4.4 g/L of MS medium for shoot generation. Seven different concentrations of IAA (0, 1, 2, 4, 6, 8, and 10 mg/L) with 4.4 g/L of MS were applied to induce root regeneration from regenerated shoots. Regenerated einkorn wheat was cultivated three different concentrations of MS media (1.1, 2.2 and 4.4 g/L) to observe development and growth, before transferring to the soil. Leaf explants did not form any callus in callus induction media. The most effective concentration of 2,4-D for callus induction from root and coleoptile was in concentrations 4 mg/L and 5 mg/L in all concentrations of MS, respectively. Shoot did not form from callus of root explant. The best shoot development from coleoptile explant of callus was in concentrations of 5 mg/L of TDZ on 4.4 mg/L of MS medium. The best root generation was 6 mg/L of IAA on 4.4 mg/L of MS medium. The highest root and shoot numbers as well as root length were in the decreased MS media concentrations, especially in 1.1 g/L of MS medium. In conclusion, einkorn wheat callus and regeneration protocol seemed to provide some valuable hints for future tissue culture, genetic studies and wheat improvement.

KEYWORDS: Coleoptile, Einkorn, *Triticum monococcum* ssp. *monococcum,* Tissue culture, Wheat.

ÖZET

SİYEZ (*TRITICUM MONOCOCCUM* SSP. *MONOCOCCUM*)
BUĞDAYININ *İN VİTRO* ÇOĞALTIMI
YÜKSEK LISANS TEZI
MEHMET ÖRGEÇ
BOLU ABANT İZZET BAYSAL ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ
BIYOLOJI ANABILIM DALI
(TEZ DANIŞMANI: PROF. DR. NUSRET ZENCİRCİ)

BOLU, NİSAN- 2019

Siyez buğdayı (*Triticum monococcum* ssp. *monococcum*) gıda endüstrisi, biyoteknolojik yaklaşım ve genetik transformasyon çalışmaları için gelecek vaadeden bir değere sahip olmanın yanı sıra arzulanan genetik karakterleri ve yüksek besin içeriği sayesinde tarımsal alanlar için de bir önem arz etmektedir. Bu çalışmanın amacı, siyez buğdayı için farklı siyez eksplant kaynakları, hormonlar ve MS konsantrastonları kullanarak etkili bir doku kültürü protokolu oluşturmaktır. Bu çalışmada, kallus oluşumu ve rejenerasyonu için siyezin kök, yaprak ve çimkını eksplant kaynakları kullanılmıştır. Kallus indüksiyonu için 5 gün *in vitro* ortamda çimlendirilmiş siyez eksplantları 10 farklı 2,4-D konsantrasyonu (0, 0.5, 1, 2, 3, 4, 5, 6, 8 ve 10 mg/L) ile takviye edilmiş, 3 farklı konsantrasyondaki (1.1, 2.2 ve 4.4 g/L) MS ortamına ekilmiştir. Ardından kalluslar, sürgün oluşumu için 6 farklı TDZ konsantrasyonundaki (0, 0.5, 1, 2, 3 ve 5 mg/L) ile 4.4g/L MS ortamına ekilmiştir. Oluşan bu sürgünlerden kök oluşumunu sağlamak için 4.4g/L MS ile 7 farklı IAA konsantrasyonu (A (0, 1, 2, 4, 6, 8 ve 10 mg/L) uygulanmıştır. Toprağa transfer edilmeden önce gelişim ve büyümenin gözlemlenmesi için, oluşmuş bu siyez buğdayı 3 farklı konsantrasyondaki MS ortamına (1.1, 2.2 ve 4.4 g/L) ekilmiştir. Yaprak eksplantları kallus indüksiyon ortamında hiç kallus oluşturmamıştır. 2,4-D'nin kök ve koleoptilden kallus oluşumu için en etkili oldığı konsantrasyon, tüm MS konsantrasyonları için sırasıyla 4 ve 5 mg/L olmuştur. Kök eksplant kalluslarından sürgün oluşmamıştır. Çimkını eksplant kalluslarından en iyi sürgün oluşumu 4.4 mg/L MS ortamı içerisinde 5 mg/L TDZ konsantrasyonu olmuştur. En iyi kök oluşumu 4.4 mg/L MS ortamında 6 mg/L IAA ile olmuştur. En yüksek kök ve sürgün sayıları ve kök uzunluğu düşük konsantrasyonlu MS ortamında, özellikle 1.1 g/L MS ortamında elde edilmiştir. Sonuç olarak, siyez buğdayı kallus ve rejenerasyon protokolunun gelecekteki doku kültürü, genetik çalışmalar ve buğday geliştirme çalışmaları için değerli ipuçları sağlayacağı görülmektedir.

ANAHTAR KELİMELER: Çimkını, Siyez, *Triticum monococcum* ssp. *monococcum*, Doku Kültürü, Buğday.

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LIST OF ABBREVIATIONS AND SYMBOLS

FAO : Food and Agriculture organizations

MS : Murashige and Skoog

NAA : Napthalenacetic acid

DKW : Driver/Kuniyuki walnut medium

NN : Nitsch and Nitsch medium

IAA : indole-3- acetic acid

IBA : indole-3- butric acide

2,4-D : 2,4-dichlorophenoxy-acetic acid

NAA : naphthalene- acetic acid

Dicamda : 3,6- dichloro-2-methoxy- benzoic acid

BAP : 6-benzyloaminopurine

2iP : 6-dimethylaminopurine

Kinetin : N-2-furanylmethyl-1H-purine-6-amine

Zeatin : 6-4-hydroxy-3-methyl-trans-2-butenylaminopurine

TDZ : thiazuron-N-phenyl-N-1,2,3 thiadiazol-5ylurea

°C : centigrade degrees

Gr : gram

L : Liter

PGR : Plant growth regulator

DMSO : Dimethyl Sulfoxide

BAP : 6-Benzylaminopurine

KIN : Kinetin

ICARDA :International Center for Agricultural Research in Dry Areas

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1. INTRODUCTION

Plants are main food source of living organisms. Energy (90%) and protein (%80) consumed by people for a living are from the plants. Today, people are approximately familiar with 250.000 plant species and, of which 3.000 of them are roughly cultivated all around the world. Cereals are among the important plant species out of those 3.000 species.

Cereal belongs to Gramineae family and its nine species have been identified, as rye (*Secale*), wheat (*Triticum*), corn (*Zea*), oat (*Avena*), millet (*Pennisetum*), rice (*Oryza*), barley (*Hordeum*), sorghum (*Sorghum*), and *triticale*. Cereals were the first agriculturally cherished plants of humankind started centuries ago (Sarwar, 2013). Today, they have still widely preferred all over the earth.

People produce a great amount of cereals grain all around the world because of their high energy than the crops. People enjoy 50% of their dailiy calories from cereals. They contain several health important nutrients for humans such as vitamins, oil, protein, and carbohydrates etc. In order to meet cereal consumption demand, cereals were produced 2650.8 million tonnes all around the world during 2017/2018 (FAO-AMIS, 2018).

Human consume cereals in two forms: raw or mostly cooked food products. Meantime, livestock and poultry used up cereals eventually for human consumption. Generally, cereals cheap to produce, easy to transport, easy to process and safe to store for longer periods. Due to all these reasons, people preferred to use and improve cereals throughout the history and wheat has become one of the most important cereal for humankind.

1.1. Wheat (*Triticum* spp.)

Wheat (*Triticum* spp.) belongs to Poaceae, grass family, tribe Triticeae. Triticum has three genome levels: diploid, tetraploid, and hexaploid. The diploid wheat species, *T. monococcum* ssp. *monococcum* and *T. urartu* are usually wild with hulled grains. einkorn wheat (*T. monococcum* ssp. *monococcum*) is cultivated in some countries mostly healthy food purpose. The tetraploids are classified as hulled (*T. turgidum* subsp. *dicoccum*) and hulledless (*T. turgidum* subsp. *durum*) wheat forms. There are five hulled hexaploid wheat species. These are *Tiriticum aestivum* subsp. spelta, *T. aestivum* subsp. *macha*, *Triticum aestivum* subsp. *aestivum* and *T. aestivum* subsp. *compactum.* Despite these five hulled hexaploid ones, only one hulledless hexaploid one is identified; *T. aestivum* subsp. *sphaerococcum* (Figure 1.1). Wheat has been estimated to be cultivated about 10.000 years ago for the first time. Moreover, diploid einkorn and tetraploid emmer wheats are known to be the earliest cultivated forms. Besides, approximately 1.000 years later, hexaploid bread wheat started to appear (Feldman, 2001).

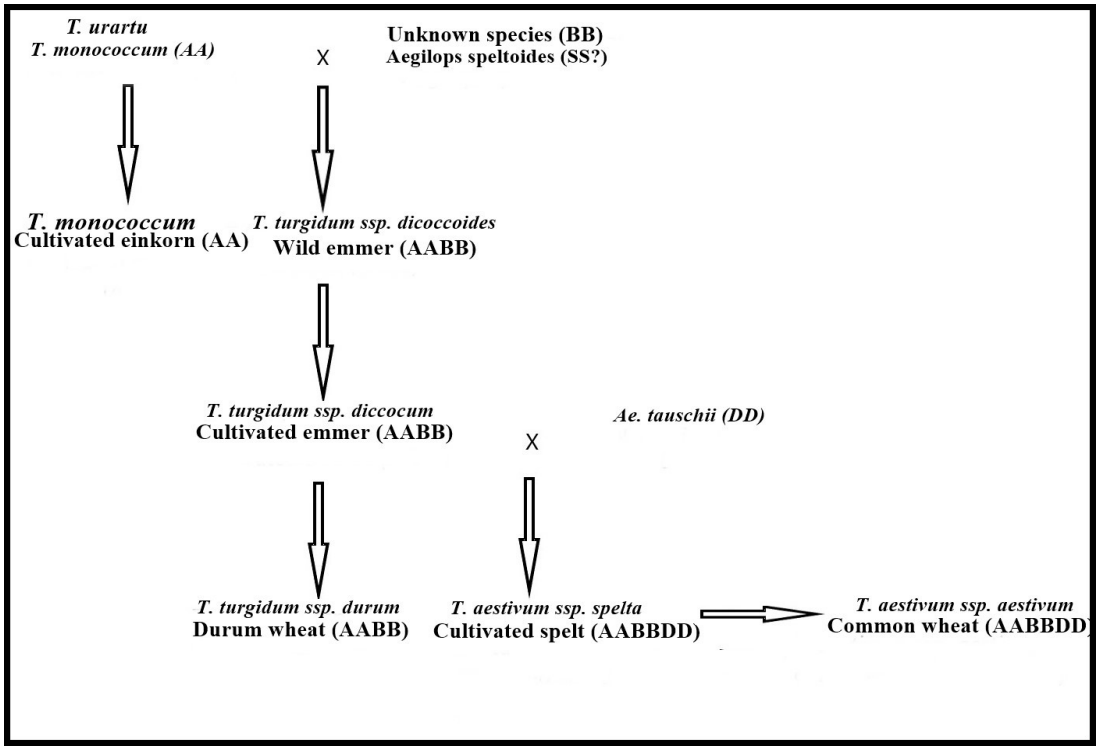

Figure 1.1. Phylogeny of domesticated species of the *Triticum* spp. (Modified from Arzani and Ashraf 2017).

Wheat is a main crop after rice and maize (Gustafson et al., 2009). It is firstly originated it the Levant region and then spread or cultivated around the world (Dinu et al., 2018). Of all cereal areas, wheat comprises 17% in temperate and Mediterranean zone as well as subtropical parts of both hemispheres (Peng, 2011). According to FAO, world wheat production is estimated around 756.7 million ton in 2017/2018 harvest season (FAO-AMIS, 2018). Turkey produced 20000000-ton wheat in 2018 (TUİK, 2018) (Figure 1.2).

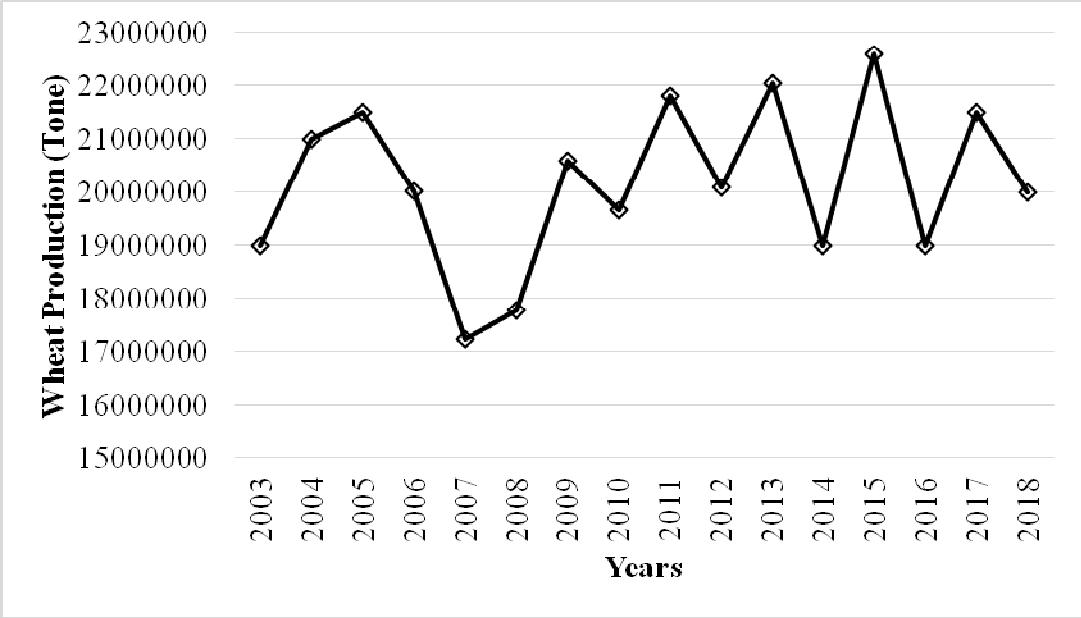

Figure 1.2. Turkey wheat production in last 15 years. (Modified from TUİK 2018 database).

As mentioned before, wheat is one of the major sources of nutrients supplying a great amount of energy for human in the world. That is why, it continues to be a strategic and indispensable food product. As shown in Figure 1.2 the total wheat production in Turkey are more unstable when compared to last 15-year production. One of the main reasons of fluctuations in yield is unexpected weather conditions, which cause yield and quality losses. Insufficient rains or heavy rains/hail adversely affected crops. Excessive seed usage is other reason for reduction in quality. In addition to these factors, cause wide fluctuations in annual production, wheat have been exposed to several diseases and pests that can cause serious damage and limit productivity

The world population is estimated to grow from 6 to 8 billion people by 2050 (Wagiran et al., 2010). Feeding this huge amount of population will be the hardest problem of humankind to face in near future. In addition, increasing yield against to several stresses like drought, salt, diseases, and aluminum toxicity scenario is unfortunately becoming difficult. To solve these problems, new strategies should be developed for high quality and higher yield with improved features such as a great tolerance/resistance to biotic and/or abiotic stresses. Conventional plant breeding systems face difficulties to produce tolerant/resistant cultivars, mainly for two reasons: shortages in gene pool and biological barrier in resistant gene transfers (Hamayun et al., 2010). Conventional plant breeding system enforced with sophisticated biotechnological approaches have a great potential for crop improvement systems. Successful plant regeneration systems from organs, tissue, and cells may, for instance, play a significant biotechnologic operational role in agronomic characters (Kowalska and Arseniuk, 2016).

Ancient wheat has protected their characteristic features over the last 12-13.000 years without any artificial interference. On the other hand, modern wheats are under discussion by the society because some health concerned issues (Dinu et al., 2018). These desired health features of ancient wheat bring it to an important point also in biotechnological studies. Einkorn (*Triticum monococcum* ssp. *monococcum*), emmer (*Triticum dicoccum Schrank*), and spelt (*Triticum aestivum* ssp. *spelta*) are the most know ancient wheats. Among them, in this study, we focused on the most common ancient wheat; einkorn.

1.1.1 Einkorn (*Triticum monococcum* spp. *monococcum*) Wheat

The diploid einkorn wheat (2n=2x=14, A^mA^m) *T. monococcum* ssp. *monococcum* was one of the first domesticated wheat in the Fertile Crescent from the wild ancestor species T. *boeticum* (Peng et al., 2011) (Figure 1.3). Karacadag Mountain is an important place in the south-east of Turkey where einkorn wheat was first domesticated (Heun et al., 1997). Then, it was spread to be cultivated around Mesopotamia. After domestication of productive hulledless wheat which can be more productive under irrigated areas had occurred, einkorn was move in to the marginal

areas faraway steppe poor mountainous lands. Over time, einkorn survived under severe environmental conditions where the others failed (Zaharieva and Monneveux, 2014).

Figure 1.3. Einkorn (*Triticum monococcum* ssp. *monococcum*) wheat, Bolu/Seben (Taken by Ayşegül Gürdal Pamuklu).

The resident names of *Triticum monococcum* ssp. *monococcum* are different according to their cultivated regions such as the Middle East, Central Asia, Europe and North-Africa (Table 1.1). Nowadays, we can see einkorn in the most rigid areas of Turkey, Caucasus, Europe, and Morocco (Zaharieva and Monneveux, 2014). In Turkey, einkorn cultivation takes place in Bolu, Kastomonu, Bilecik, Sinop, Balıkesir, and Çankırı. The aim of einkorn use differs depending on cultural, social, economic status and technological levels of the countries. As an example, main consumption type of einkorn in Turkey is as bulgur or animal feed.

Table 1.1. The resident names of *T. monococcum* ssp. *monococcum* in countries.

Language	Resident names
Turkish	IZA, kaplıca buğdayı, siyez
Moroccan	Skaliah
Italian	Spelta minore, monococco
Greek	Tiphe
English	Einkorn, small spelt
German	Einkorn, kleiner spelz
Bulgarian	Ednozarnest limetz
Croatian	Jednozrnac
Danish	Enkorn
Hungarian	Alakor

Einkorn grain has charming nutritional value. Protein, carotenoids and minerals of einkorn are higher than bread wheat (Abdel- Aal et al., 1995; Brandolini et al., 2008). In addition, it has higher amount of soluble sugar and minerals and less total insoluble dietary fibers than those of other cultivated wheats (Abdel- Aal et al., 1995). Einkorn wheat grain has more zinc, iron, copper and selenium than bread wheat grain (Suchowilska et al., 2012). Comparing einkorn wheat with bread and durum wheat for protein content, einkorn has higher protein (Zaharieva and Monneveux, 2014). Einkorn wheat grain has between 13.2-22.8% protein. Einkorn grain also, includes less α amylase inhibitors than durum and bread with and eases to digestion (Vitozzi and Silano, 1976). Carotenoids are two times higher in einkorn than modern they are in wheats (Grausgruber et al., 2010). Einkorn has approximately three to four times higher lutein, four to five times higher riboflavin and higher concentration in pyridoxine. In addition to these, einkorn contains higher phosphorous, vitamin B6, yellow pigments, and potassium (Abdel-Aal et al., 1995).

Einkorn wheat has also an important value for health. For example, under favorable lutein level in einkorn, einkorn was reported to exert protective effect against age-related macular degeneration, which is a vision related with disease (Zaharieva and Monneveux, 2014). It may also be effective against cardiovascular diseases. For instance, it was indicated that a steroid compound of einkorn could reduce the levels of cholesterol (O'Neill et al., 2004). We are all aware that cancer incidence has recently increased rapidly throughout the world. Einkorn was found, as well, to be effective against the several cancers such as lungi stomach, ovary and breast (Woyengo et al., 2009). Furthermore, einkorn has many valuable features against to the wheat fungal diseases (Singh et al., 2008) and highly resistant to pests and other wheat diseases (Konvalina et al., 2010).

Einkorn is treasure for wheat improvement programs because of its great reservoir of genes (Monneveux et al., 2001). It is already used in several breeding programs to most extent: for example, for durum wheat improvement against earliness and rust resistance at International Center for Agriculture in Dry Areas (ICARDA) and against biotic stresses in Martonvasar, Hungary (Zaharieva and Monneveux, 2014).

1.2 Tissue Culture

In the last a few decades, when the amount and the feature of the production were considered cereals were developed relatively slow by applying classical (traditional) breeding techniques. Scientists have to work with thousands of plants and are also dependent on plant's growth period at field. However, working in cell level is not rested on the growth period in an *in vitro* study.

Classical breeding techniques possess some inhibitions while it has some advantages as well. For example, it needs time to success and, it has also utilized a constrained gene pool via restricted techniques for breeding programs (Malik et al., 2003). Modern biotechnology such as tissue culture, genetic engineering, and genetic transformation provide a new array to improve wheat cultivars. Genetic transformation offers a good potential to improve wheat for a better yield and quality, nonetheless genetic transformation needs an efficient, a reliable, a stable, and a reproductive tissue culturing system (Khan et al., 2015). One important step in biotechnological research on wheat needs reliable callus induction and efficient regeneration protocol (Sikandar et al., 2007).

The plant tissue culture is a different method used for micropropagation, callus formation, secondary metabolites production, plant conservation, plant improvement and disease elimination, using small pieces of tissue called as explants to produce continuously thousands of plants in a short time regardless of the season and weather conditions throughout the year. (Jabeen et al., 2016). It also, increases the natural levels of *in vitro* production of important products and large amounts of plant materials (Pande et al., 2013).

Callus culture is commonly used in plant biotechnology. Especially, in monocot plant species callus culture is a very important step since the plant regeneration is hard to achieve. For this reason, callus induction, callus proliferation, and callus growth are very significant steps in wheat tissue culture studies.

Callus is an undifferentiated cell forms which has the ability to swiftly proliferate. In order for the formation of callus cells, various types of explant and

specific plant growth regulators are needed (Naqvi et al., 2002). Callus comes out from the differentiated cell. Almost all cells of callus tissue are totipotent which can produce a whole plant (Nagata and Takebe, 1971) and can provide somatic embryogenesis under certain conditions (Steward et al., 1958). Callus is classified into different groups based on their morphological characters. If there is no organ formation on callus structure, these kind of callus types are called as a friable or compact callus. On the other hand, any organ regeneration on callus structure are called shooty, rooty and embryonic callus depending on the organ regeneration level (Frank et al., 2000) (Figure 1.4). Differentiation and dedifferentiation of callus structures depend on the auxin and cytokinin hormone types and their concentrations.

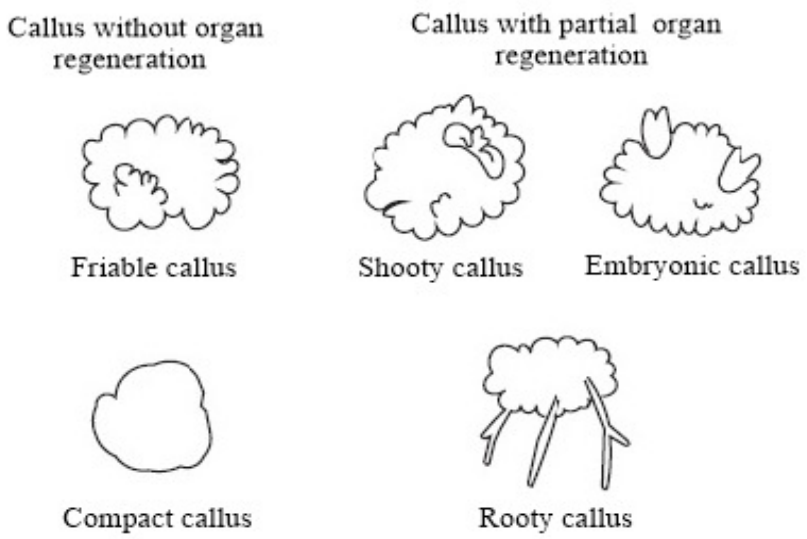

Figure 1.4. Different types of callus structure (Modified from Ikeuchi et al., 2013).

For better understanding of tissue culture in plants, informations about factors which effect on callus induction and plant regeneration is highly important. These factors include genotypes, explant types, growth media, plant growth regulators, and explant sterilization.

1.2.1 Genotype

Plant genotype is very important and it is well documented to understand its influence on the callus induction and regeneration of major cereals (Özgen et al.,

1968). It is certain that wheat tissue culture response polygenic in nature (Bregitzer and Cammplel, 2001). This genetic information can be transmitted by heredity (Chevrier et al., 1990). Different wheat species reflected different responses under the same tissue culture conditions (Jabeen et al., 2016). Genotypes have some great effect on the callus induction, the embryonic callus formation, and the plant regeneration (Fennel et al., 1996; Aydın et al., 2011). Because of the all these reasons each plant species can have a specific *in vitro* protocol.

1.2.2 Explant Type

One of the main factors to achieve efficient tissue culture protocol is choosing a favourable explant type (Mahmood and Razzaq, 2017). Various types of explants such as shoot tips, glumella, lemma, inflorescence stem section, anters, nodes, shoots apical meristem, and mature and immature embryos have been extensively used for the callus induction and regeneration processes (Viertal and Hess, 1996; Özgen et al., 1996; Lu, 1988; Lu, 1992; Benkirane et al., 2000; Konieczny et al., 2003; Haliloğlu et al., 2006) Explant types highly affect the callus induction and the regeneration frequencies in wheat (Redha and Talaat, 2008). Generally, immature and mature embryos are more efficient than other explant types for callus formation and regeneration.

The immature embryo is the most used explant source for wheat in *in vitro* culture application by reason of its high callus production ability and regeneration percentage (Vendruscolo et al., 2008; Gill et al., 2014; Yang et al., 2015). On the other hand, immature embryo generation has some limitations such as larger laboratory area requirement, generation time, and intensive workload (Mahmood and Razzaq, 2017). The other most used explant type is the mature embryo. Mature embryo has less percentage of callus and regeneration rate than immature embryo (Özgen et al., 1998). However, mature embryo has some advantages over the immature embryo. Mature embryos are easy to handle without any time limitation, and it can be stored for a long time (Kowalska and Arseniuk, 2016).

Another factor related to influence of tissue culture is explant age which can decrease the callusing percentage and regeneration capacity (Khan et al., 2015).

1.2.3 Growth Media

The growth media structure plays an essential role in tissue culture (Tamas et al., 2004). The tissue culture medium contains macronutrients and micronutrients, including amino acids, vitamins, carbon source organic supplements, solidifying agents and plant growth regulators. Different types of growing media such as B6, N6, NN, DKW, and MS are used to cultivate various plant. MS, includes double concentration of macro elements, is one of the most preferred tissue culture media because of its higher embryonic callus formation property (Carman et al., 1987).

Other two important tissue culture media elements are sugars as a carbon sources and gelling agents. In plants, carbohydrates play an important role in several functions, they used as a substrate in respiration, and production pathway many macromolecules. Sucrose and maltose are generally used as a sugar source affects the formation of somatic embryogenesis, generation of auxiliary buds and adventitious roots and regeneration of plants (Vinterhalter and Vinterhalter, 1997; Khan et al., 2015).

Different gelling agent used for solidification in tissue culture medium. One of the most used gelling agents in the tissue culture is agar, a polysaccharide, obtained from seaweeds (Liang et al., 1987). Agar is mainly used for preparing semi-solid and solid media because it does not react with media content and it shows resistance to digestive plant enzymes.

1.2.4 Plant Growth Regulators

Plant growth regulators are indispensable for a successful in vitro study (Gaspar et al., 1996). Dosage and combination of plant growth regulators are very

vital to achieve regeneration (Kouassi et al., 2017). Their dosage and combination can be changeable among genotypes and explant types.

Naturally occurring plant hormones can be divided into six main groups such as auxins, cytokinins, abscisic acid, ethylene, gibberellins and brassinosteroids Among them, the most important hormones in the growth regulation and the developmental stage organizations such as callus and plant regeneration in the tissue culture are auxins, cytokinins, and their combinations (Mahmood et al., 2012). Other hormones, including abscisic acid, gibberellins, ethylene etc. are essential for plant growth and regulation but they do not directly affect the plant growth and development; in other words, they are active but they do not have a pioneering role (Gaspar et al., 1996).

Auxin is widely used in tissue culture studies as a part of nutrient media. The efficiency of auxins is well documented on the several plant growth processes. For example, they acidify cell wall, provide cell growth, initiate cell division, promote vascular differentiation, induce root formation and as well callus. The most commonly used auxins in *in vitro* culture of plants are IAA, IBA, 2,4-D, NAA, and dicamba. Among them, IAA is the only hormone naturally synthesized in tissues. (Guan et al., 1997). Auxins are mostly needed for callus and root induction from explant. For callus induction 2,4-D and for root induction IAA are the most preferred auxin types.

The most commonly used cytokinins in tissue cultures are BAP, 2iP, KIN, Zeatin, and TDA respectively. Zeatin and 2iP are two important naturally synthesized cytokinins. The main functions of cytokinins in tissue cultures are cell division stimulation, shoot formation induction, and axillary shoot proliferation. In cell division, auxin and cytokinin effect the cell cycle in a different phase (Gasper et al., 1996). Therefore, it is important to balance the levels of auxins and cytokinins. Auxin/cytokinin ratio also affect the organ formation in plant tissue culture. If auxin to cytokine ratio is high, root formation is induced. However, is low, shoot induction is promoted (Skoog and Miller, 1957).

1.2.5 Explant Sterilization

In plant tissue culture, different parts of plants are basically transferred to a suitable medium where they may grow. The most important thing in that process for healthy production is the sterilization of explants such as cell, tissue and/or organs, and nutrient medium (Odutayo et al., 2004). During experimental *in vitro* studies, contamination is the most serious problem which causes to lose explants by microorganism. Virus, bacteria, fungi, and yeast are microorganisms can cause contamination in *in vitro* studies (Örgeç et al., 2018). Presence of these organisms in nutrient medium results reducing shoot proliferation, tissue necrosis, growth lateness, and root growth reduction (Nadha et al., 2012).

There are several sterilant agents used in plant tissue culture: hydrogen peroxide, sodium hypochlorite, ethanol, mercuric chloride, and Tween 20 (Ishfag et al., 2016). Sterilants concentration and exposure time should be chosen carefully because they can be toxic on the explant. Therefore, for each plant species different sterilization protocols should be used. The success in the culture is primarily dependent on an effective sterilization protocol with less contamination and higher germination ratio.

2. AIM AND SCOPE OF THE STUDY

Einkorn wheat (*Tiriticum monococcum* ssp. *monococcum*) is one of the first cultivated wheat in the Fertile Crescent. It has valuable genetic characters such as biotic/abiotic stress and disease resistance which may be useful in breeding programs and be benefited for improvement of wheat cultivars. In addition, some genetic transformation studies can be planned near future to increase yield potential of einkorn wheat. Successful in breeding, genetic engineering, and genetic transformation studies, in that context, need to have an efficient tissue culture protocol.

For this reason, in the present study, we aim that is to determine an efficient tissue culture protocol for einkorn (*Triticum monococcum* ssp. *monococcum*) wheat. In order to provide and efficient tissue culture protocol, different explant types such as coleoptile, root, and leaf were tested in three different concentrations of MS growing media with 10 different 2,4-D concentrations for callus induction. Six different TDZ and seven IAA hormone concentrations were also used for regeneration protocol after callus induction.

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3. MATERIALS AND METHODS

3.1 Seed Source and Experiment Site

In this study, einkorn wheat from 2014-2015 İhsangazi / Kastamonu harvest was used as a seed source. The experiments were conducted at the Laboratory of Plant Genetics & Pathology, Department of Biology, University of Bolu Abant İzzet Baysal, Bolu, Turkey.

3.1.1 Labware Sterilization

In vitro studies must be in aseptic conditions to avoid contamination. For this reason, all labware must be sterile before each experiment. In this study, beaker, pens, strainer for seed washing, scalpel, and magenta box were sterilized in an autoclave (Nuve- OT 40L) at 120 °C for 15 minutes. During the culturing in the laminar air-flow (Nuve- LN 090), forceps and scalpel were sterilized with 70% (v/v) ethanol. Scalpel blades and petri were bought in a sterile package.

3.1.2 Sterile Water Preparation

Distilled water was used for seed washing and culture preparation. For sterilization, distilled water was autoclaved at 120 °C for 15 minutes.

3.1.3 Seed Sterilization

For seed sterilization, dehulled grains of einkorn seeds were put into 100 ml distilled water contain five drops of Tween20 (Merck) and then were mixed for one minute using magnetic stirrer. After one minute, seeds were washed with sterile water. Then, all seeds were put into 40% commercial bleach (Domestos / 4.6%

NaClO) and mixed for 15 min using magnetic stirrer. After 15 minutes, seeds were washed with sterile water until removing Domestos (Figure 3.1) (Örgeç et al., 2018).

Figure 3.1. Seed sterilization and culturing process. **A:** Hulled einkorn wheat. **B:** Dehulled einkorn wheat. **C:** Sterilization process. **D:** Sterilized seed in germination media.

3.2 Explant Source

Immature and mature embryos are the most preferred explant types in wheat tissue culture although they have some advantages and disadvantages as we mentioned before. In this study, root, coleoptile, and leaf were used as an explant source to determine the most effective explant type for einkorn wheat.

3.3 Climate Room and pH

Each step of *in vitro* propagation of einkorn wheat, except callus culture, all cultures were kept in the climate room with 16 h light / 8 h dark period at 24 ± 2 ℃. Callus cultures were incubated only in dark at 24 ± 2 ℃ (Benkirane et al., 2000). In

all media preparation, pH was adjusted at 5.7-5.8 using 1 N HCI and 1 N NaOH (Arshad et al., 2013).

3.4 Media Preparation

During *in vitro* experiment MS (Duchefa Biochemie B.V, Netherlands) medium was used as an essential nutrient media. Sucrose (Duchefa) and plant agar (Duchefa) were used as a carbon source and solidification agent, respectively. 2,4-D (Sigma), IAA (Duchefa), and TDZ (Duchefa) were used for callus induction and regeneration.

To prepare a 1 mg/ml stock solution of 2,4-D, 100 mg of 2, 4- D was put in a test tube and 2-5 ml of ethanol was added to dissolve the powder. Then, the solution was gradually diluted with 100 ml distilled water. The stock solution was stored in a refrigerator. 1.0 ml of the stock solution was added to 1 liter of medium to obtain a final concentration of 1.0 mg/L.

The case was similar with IAA and TDZ. IAA hormone, was dissolve in ethyl alcohol or 1N NaOH and the same procedure above was followed. The IAA were stored in amber bottles or in black paper covered bottles. IAA were kept in the dark since they were unstable under the light. The same procedure was applied for TDZ hormone except DMSO was used for dissolving it.

3.4.1 Germination Media Preparation

For preparation 1-liter germination medium;

- 4.4 g/L of MS was weighed and placed in a 1-liter autoclave bottle,
- 25 g/L sucrose and 750 ml distilled water were added into each bottle. A magnetic stirring bar was put into the bottles and stirred by a magnetic stirrer until dissolved,
- Distilled water was added to complete the whole solution for 1-liter,
- pH of germination media was adjusted to 5.7-5.8,

- 7.5 g/L agar was added to the solution,
- The bottle was half closed and put into autoclave machine at 120 °C for 15 minutes for sterilization,
- End of the sterilization, the solution was taken into the laminar air-flow and poured in petri (each petri had around 25 ml solution),
- After cooling and solidification, seeds were cultured into media (each petri had ten seeds) in laminar air flow and petri closed with parafilm,
- All cultures were kept at climate room for five days (Benkirane et al., 2000).

3.4.2 Callus Induction Media Preparation

Callus induction media preparation was the following;

- Different concentrations of three MS solution of 4.4 g/L, 2.2 g/L, and 1.1 g/L respectively were weighed and placed in three different 1-liter autoclave bottles,
- 25 gr sucrose and 750 ml distilled water were added into each bottle. A magnetic stirring bar was put into the bottles and stirring by a magnetic stirrer continued until dissolved,
- Distilled water was added to complete the whole solution up to one litter for each bottle,
- Each three MS solutions were divided into 10 different 100 ml autoclave bottles,
- Different MS solution concentrations were supplemented with 10 different 2,4-D doses of, 0, 0.5 mg/L, 1 mg/L, 2 mg/L, 3 mg/L, 4 mg/L, 5 mg/L, 6 mg/L, 8 mg/L, and 10 mg/L, respectively,
- pH of callus induction media was adjusted at 5.7-5.8,
- 0.75 g/L agar was added to each 100 ml solution bottles,
- The bottles were half closed and put into autoclave machine at 120 °C for 15 minutes for sterilization,
- After the sterilization, the solutions were taken into the laminar air flow and poured in petri (each petri had around 25 ml solution),

- After cooling and solidification, root, coleoptile, and leaf were cut from germinated seed and were cultured into media in laminar air-flow and petri closed with parafilm,
- Each 2,4-D combination had three petri and each petri had five explants from each explant types,
- All cultures were kept in the climate room for 6 weeks in dark conditions (Benkirane et al., 2000; Kowalska and Arseniuk, 2016),
- Every 15 days we took subcultures for in all media with the same callus induction media (Zale et al., 2004).
- After five-six weeks, callus formation percentage and size data were recorded.

3.4.3 Shoot Induction Media Preparation

Shoot induction media preparation follows;

- 2.64 g/L of MS was weighed and placed in a one-liter autoclave bottle,
- 15 g/L sucrose and 400 ml distilled water were added into the bottle. A magnetic stirring bar was put into the bottle and was stirred by a magnetic stirrer until sucrose and MS dissolved,
- Distilled water was added to complete the whole solution up to 600 ml,
- The whole solution was divided into six and put in to 100 ml autoclave bottles,
- Solutions were supplemented with six different TDZ dose 0, 0.5 mg/L, 1 mg/L, 2 mg/L, 3 mg/L, 5 mg/L, respectively,
- pH of shoot induction media was adjusted at 5.7-5.8,
- 0.75 g/L agar was added to each 100 ml solution bottles,
- The bottles were half closed and put into autoclave machine at 120 °C for 15 minutes for sterilization,
- After the sterilization, the solutions were taken into the laminar air-flow and poured in petri (each petri had around 25 ml solution),

- After cooling and solidification, calli were cultured randomly into shooting media,
- Each TDZ combination had three petri and each petri had three calli,
- All cultures were kept in the climate room for 6 weeks (Benlioğlu and Birsin, 2017),
- Every 15 days we took subcultures for all media with the same shoot induction media,
- After six weeks, mean frequency (%) of explants developed shoots and mean number of shoots per callus were recorded.

3.4.4 Root Induction Media Preparation

Root induction media preparation is the following;

- 3.08 g/L of MS was weighed and placed in a one-liter autoclave bottle,
- 17.5 g/L sucrose and 500 ml distilled water were added into bottle. A magnetic stirring bar was put into the bottle and stirred by a magnetic stirrer until MS and sucrose dissolved,
- Distilled water was added to complete the whole solution at 700 ml,
- The whole solution was divided into seven and put in to 100 ml autoclave bottles,
- Solutions were supplemented with 7 different IAA doses of 0, 1 mg/L, 2 mg/L, 4 mg/L, 6 mg/L, 8 mg/L, and 10 mg/L, respectively,
- pH of root induction media was adjusted at 5.7-5.8,
- 0.75 g/L agar was added to each 100 ml solution bottles,
- The bottles were half closed and put into autoclave at 120 °C for 15 minutes for sterilization,
- After the sterilization, the solutions were taken into the laminar air-flow and poured in petris (each magenta bottle had around 30 ml solution),
- After cooling and solidification, shoot regenerated calli were cultured randomly into rooting media,

- Each IAA combination had three petri and each petri had three shoots regenerated calli,
- All cultures were kept in the climate room for four weeks,
- Every 15 days we subcultured all media with the same root induction media,
- After four weeks, the percentage of shoots that rooted, the mean number of roots per rooted shoots, and mean length of longest roots were documented.

3.4.5 Root and Shoot Growth Media Preparation

Root and shoot growth media preparation follows;

- Different concentrations of three MS solutions of 2.2 g/L, 1.1 g/L, and 0.55 g/L respectively were weighed and placed in three different one-liter autoclave bottles,
- 12.5 gr sucrose and 300 ml distilled water were added into each bottle. A magnetic stirring bar was put into the bottles and run in a magnetic stirrer until MS and sucrose dissolved,
- Distilled water was added to complete the whole solution to 500 ml in each bottle,
- pH of root and shoot growth media was adjusted at 5.7-5.8,
- 3.75 g/L agar was added to each three solutions,
- The bottle was half closed and put into autoclave machine at 120 °C for 15 minutes for sterilization,
- After the sterilization, the solutions were taken into the laminar air flow and poured in petris (each magenta bottle had around 30 ml solution),
- After cooling and solidification, regenerated plants were cultured into media in laminar air flow and petri closed with parafilm,
- All cultures were kept in the climate room for 15 days,
- After 15 days, mean number of roots and shoots, mean longest length of root and shoot length data were collected.

3.4.6 Acclimatization

After 15 days, healthy regenerated plants were taken from the root and shoot growing medium and washed carefully with sterile water to clean any adhering. To decrease any contamination, pots were also washed by sterile water and dried. The pots filled with soil and vermiculite (Sigma, ratio respectively; 2:1) then regenerated einkorn plants were carefully sown into the pots. All pots were covered by polythene bags and incubated in the climate room. After 5-6 days we opened some spots on the polythene bags and one week later we took all polythene bags off.

3.4.7 Statistical Analysis

Data were analyzed using SPSS version 25.0 software. For statistical analysis, analysis of variance (ANOVA) with Duncan post hoc test were used to compare differences in the mean of callus induction and plant regeneration rate *in vitro*. Each experiment was repeated three times. Differences among means yielding a probability of $P < 0.05$ were considered statistically significant (Saha et al., 2017).

4. RESULTS

4.1 Explant Type

The callus formation and regeneration potential of einkorn *(Triticum monococcum* ssp. *monococcum*) wheat were investigated by using different explant types; root, coleoptile, and leaf. Fifth days of germination, each explant was cultured on callus induction media.

All leaf explant died after they transfer to callus induction media. Root explants gave good result on callus induction media, however, calli produced from root explants did not generate any shoot on regeneration media. Coleoptile explants were good for callus induction and during regeneration stage. All regenerated plants were from the coleoptile explant.

4.2 Callus Induction

Callus induced from root and coleoptile explants was visible within a week on the cutting end of the explants while, leaf explants did not produce any callus structure. After 42 days of callus formation, frequency as a percentage of explant producing cali were calculated and their sizes were observed for both explant types. Callus percentage were between 20% - 100% for all treatment and embryonic callus never formed in both explant types.

In order to investigate the effects of concentration differences of growing medium and hormone on callus induction, three different MS concentration with ten different concentrations of 2,4-D were applied.

The effect of different concentrations of MS and 2,4-D on callus formation from root explant was shown in Figure 4.1. Callus induction started in 4 - 5 days after cultured on callus induction media from root explant. After each subculture mass of callus were increased. Callus from root explant had the white color (Figure 4.2).

Among all treatment, 2.2 g/L of MS contain 8 mg/L of 2,4-D was statistically significant for callus formation. 2.2 g/L of MS was significantly increased the callus formation from root explant when compared to 1.1 g/L and 4.4 g/L of MS.

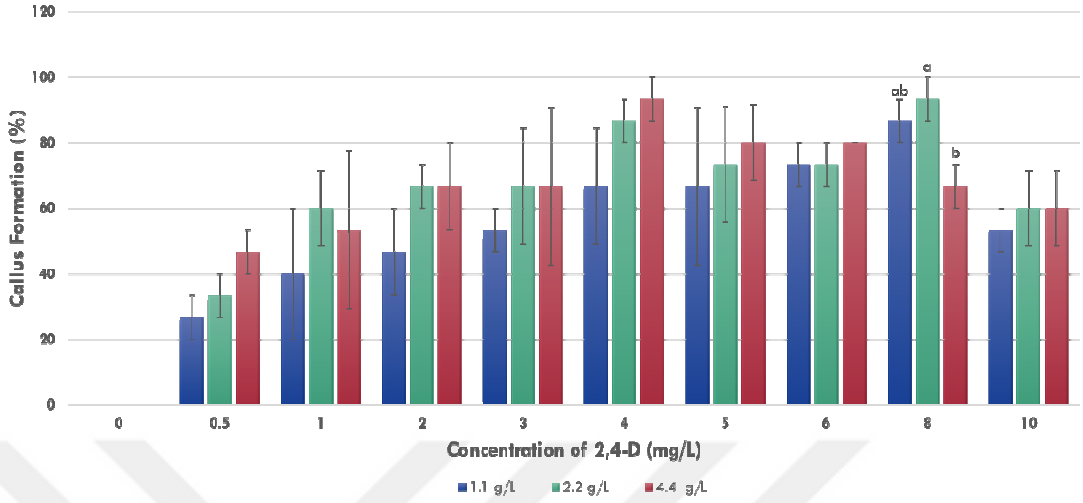

Figure 4.1. The effect of different MS concentrations on callus formation at same 2,4 D concentration from root explant. Different letters indicate significant differences at $p < 0.05$.

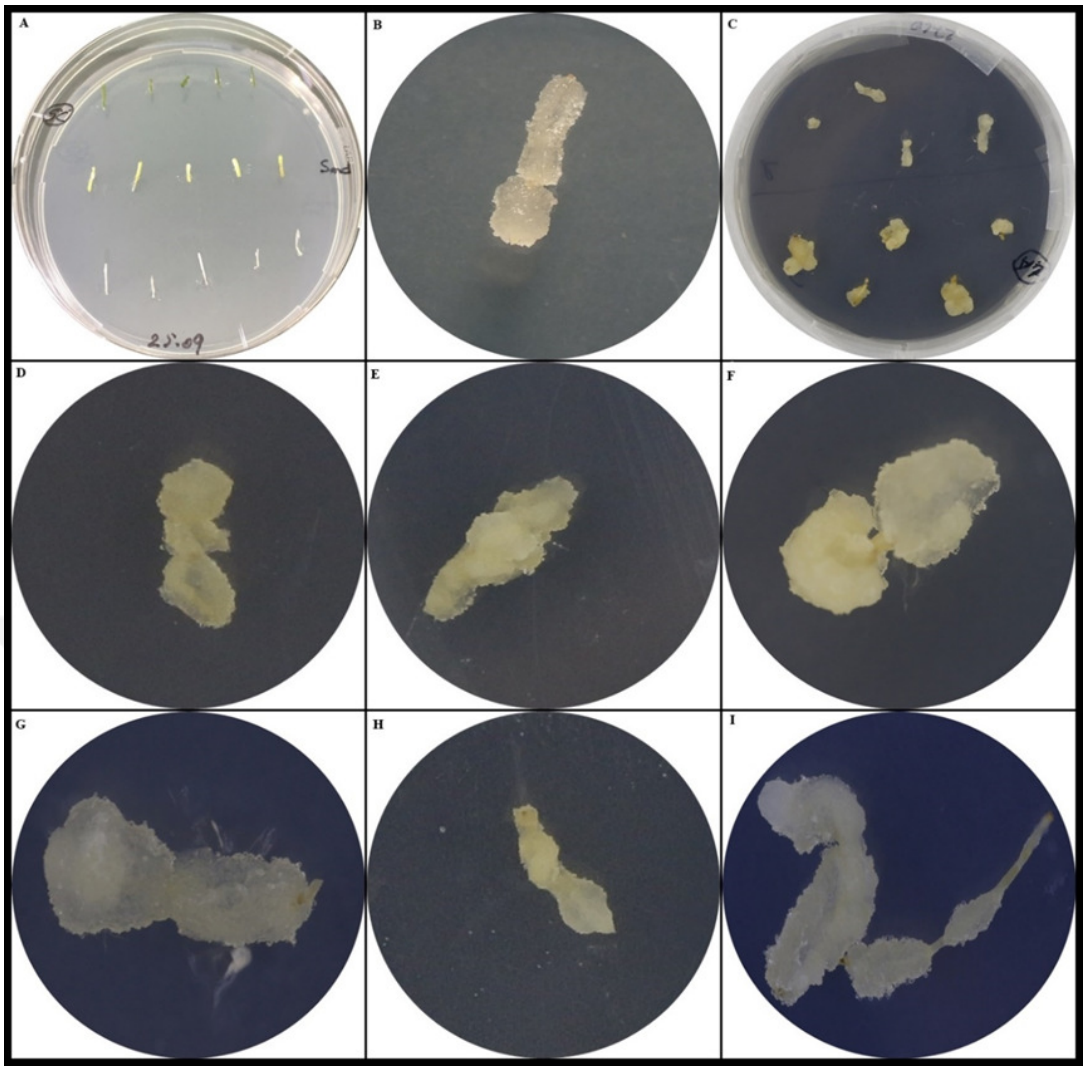

Figure 4.2. The effect of 2,4-D and different amounts of MS medium on the callus induction from root explant. **A:** First day of cultured all explants in callus induction media. **B:** 9 days of callus induction on 1.1 g/L of MS at 1 mg/L of 2,4-D. **C:** 30 days of callus induction from root and coleoptile explants on 4.4 g/L of MS at 3 mg/L of 2,4-D. **D:** 30 days of callus induction on 2.2 g/L of MS at 6 mg/L of 2,4-D. **E:** 30 days of callus induction on 2.2 g/L of MS at 1 mg/L of 2,4-D. **F:** 30 days of callus induction on 1.1 g/L of MS at 1 mg/L of 2,4-D. G: 30 days of callus induction on 4.4 g/L of MS at 3 mg/L of 2,4-D. **H:** 30 days of callus induction on 4.4 g/L of MS at 10 mg/L of 2,4-D. **I:** 42 days of callus induction on 2.2 g/L of MS at 6 mg/L of 2,4-D.

4.4 g/L of MS medium contain 4 mg/L 2,4-D, root explant significantly produced the highest callus formation percentage with 93%. On the other hand, callus induction was significantly the lowest (47%) when explant cultured on 0.5 mg/L of 2,4-D in 4.4 g/L of MS media. Callus sizes were the largest at 4 mg/L of

2,4-D for 4.4 g/L of MS media while the smallest at 0.5 mg/L, 1 mg/L, 2 mg/L, and 10 mg/L of 2,4-D for root explant (Table 4.1).

In 2.2 g/L of MS media contained 8 mg/L of 2,4-D, root explant significantly produced the highest callus formation with 93%. On the other hand, 0.5 mg/L of 2,4-D led the lowest callus formation percentage with 33%. When 0.5 mg/L, 1 mg/L, 2 mg/L and 3 mg/L of 2,4-D, compared to other 2,4-D concentrations the smallest callus sizes were detected in former concentrations (Table 4.1).

In 1.1 g/L of MS media, results shown that 8 mg/L of 2,4-D produced significantly the highest callus formation percentage with 87%. On the other hand, 0.5 mg/L of 2,4-D caused to the lowest callus formation percentage with 27%. Callus size score shown that 1 mg/L, 4 mg/L, and 8 mg/L of 2,4-D caused the largest callus sizes, but 3 mg/L of 2,4-D resulted in the smallest callus sizes in 1.1 g/L MS (Table 4.1).

Table 4.1. Effect of different concentrations of 2,4-D and MS media on callus formation from root explant.

Influence of MS media and 2,4-D on root explant callus induction						
Auxin (2,4-D) (mg/L)	**4.4 g/L MS**		**2.2 g/L MS**		**1.1 g/L MS**	
	Callus percentage[t]	**Callus size**	**Callus percentage[t]**	**Callus size**	**Callus percentage[t]**	**Callus size**
0	0	0	0	0	0	0
0.5	47^{bcde}	++	33^{def}	++	27^{ef}	++
1.0	53^{abcde}	++	60^{abcde}	++	40^{cde}	+++
2.0	67^{abcde}	++	67^{abcde}	++	47^{bcde}	++
3.0	67^{abcde}	+++	67^{abcde}	++	53^{abccde}	+
4.0	93^{a}	++++	87^{ab}	+++	67^{abcde}	+++
5.0	80^{abc}	+++	73^{abcd}	+++	67^{abcde}	++
6.0	80^{abc}	+++	73^{abcd}	+++	73^{abcd}	++
8.0	67^{abcde}	+++	93^{a}	+++	87^{ab}	+++
10.0	60^{abcde}	++	60^{abcde}	+++	53^{abccde}	++

[t] Different letters indicate significantly differences at $p < 0.05$.
+: Tiny, ++: Small, +++: Medium, ++++: Large.

The effect of different concentrations of MS and 2,4-D on callus formation from coleoptile explant was shown in Figure 4.3. At the concentration of 10 mg/L of 2,4-D, there were no significant differences in the callus formation among the different concentration of MS. In the case of 1 mg/L of 2,4-D concentration, the 4.4 g/L of MS resulted in higher callus formation than 2.2. g/L of MS. 4.4 g/L of MS caused the highest callus formation on coleoptile explant. It was interesting that 2.2

g/L of MS resulted in lower callus formation than that of 1.1 g/L of MS at the concentration of 0.5 mg/L, 1 mg/L, 2 mg/L, and 3 mg/L of 2,4-D. In the concentration of 0.5 mg/L 3 mg/L, 4 mg/L, 6 mg/L, 8 mg/L of 2,4-D, MS concentration of 4.4 g/L caused significant increase in callus formation of coleoptile when compared to the other MS concentrations. At the concentration of 5 mg/L of 2,4-D, 4.4 g/L of MS led to significantly higher callus formation on coleoptile only than 1.1 g/L of MS.

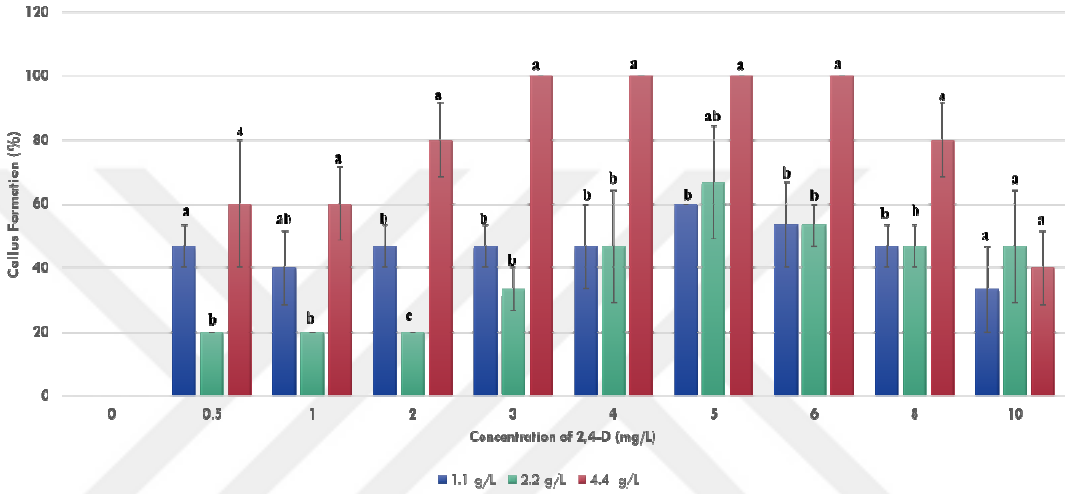

Figure 4.3. The effect of different MS concentrations on callus formation at same 2,4 D concentration on coleoptile. Different letters indicate significant differences at $p < 0.05$.

Callus induction started in 4 - 5 days after cultured coleoptile explant on callus induction media. Day by day, callus color started to turn from light brown to white. Callus mass was also increased after each subculture (Figure 4.4 and Figure 4.5).

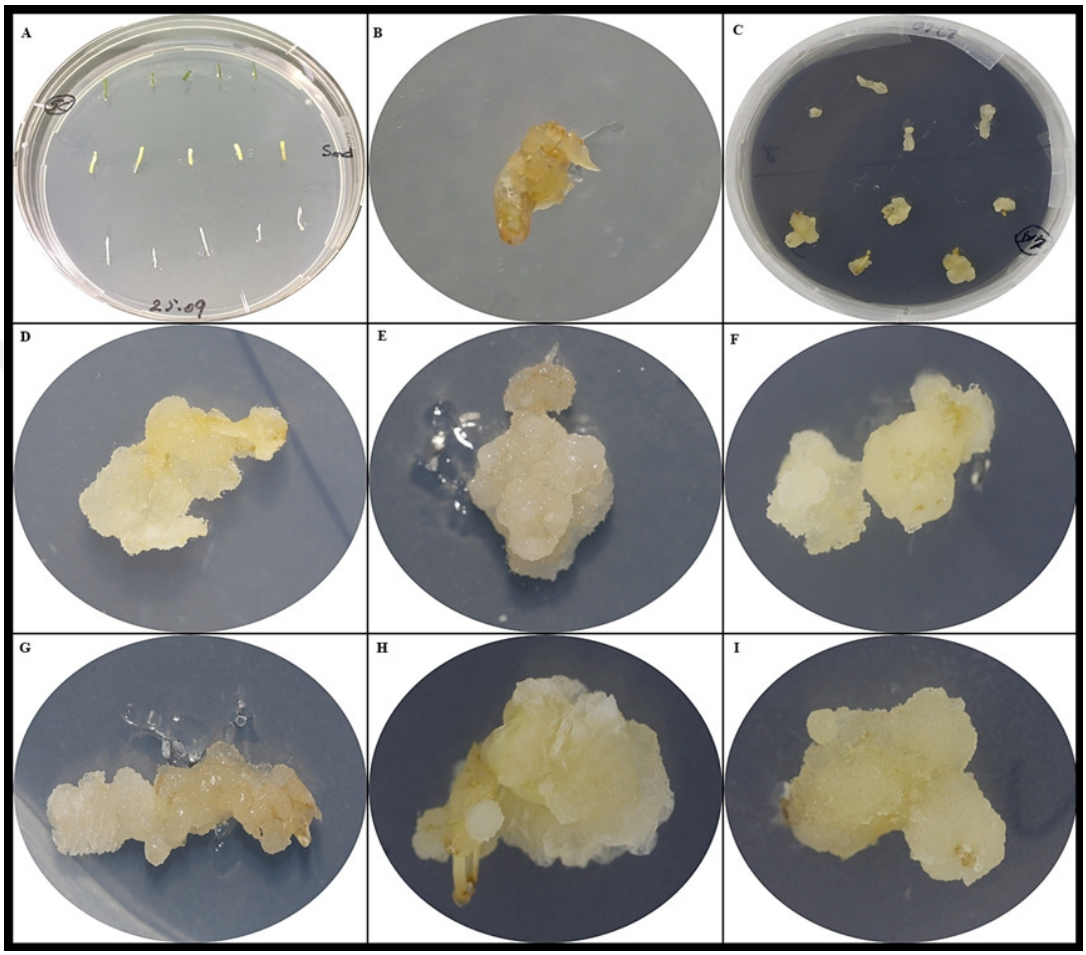

Figure 4.4. The effect of 2,4-D and different concentrations of MS medium on callus induction from coleoptile explant. **A:** First day of cultured all explants in callus induction media. **B:** 9 days of callus induction on 1.1 g/L of MS at 1 mg/L of 2,4-D. **C:** 30 days of callus induction from root and coleoptile explants at 3 mg/L of 2,4-D on 4.4 g/L of MS. **D:** 30 days of callus induction on 2.2 g/L of MS at 4 mg/L of 2,4-D. **E:** 30 days of callus induction on 4.4 g/L of MS at 1 mg/L of 2,4-D. **F:** 30 days of callus induction on 1.1 g/L of MS at 1 mg/L of 2,4-D. **G:** 30 days of callus induction on 4.4 g/L of MS at 6 mg/L of 2,4-D. **H:** 30 days of callus induction on 4.4 g/L of MS at 5 mg/L 2,4-D. **I:** 30 days of callus induction on 4.4 g/L of MS at 3 mg/L of 2,4-D.

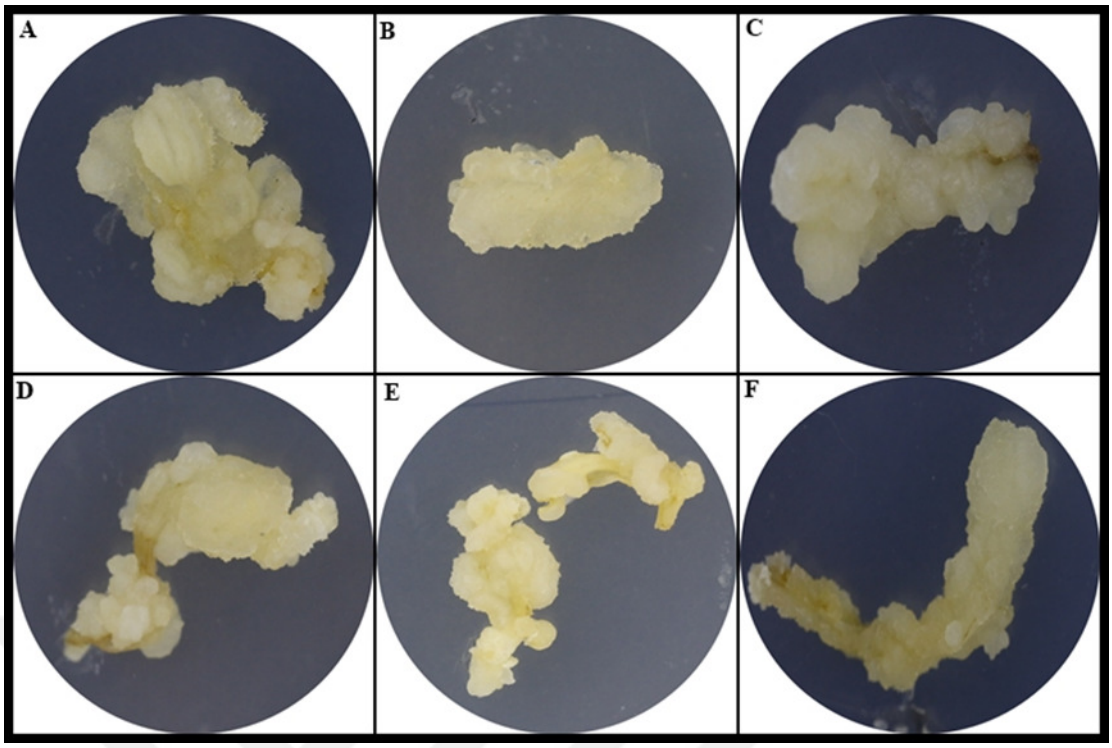

Figure 4.5. The effect of 2,4-D and different amounts of MS medium on the regenerable callus induction from coleoptile explant. **A:** 42 days of callus induction on 1.1 g/L of MS at 4 mg/L of 2,4-D. **B:** 42 days of callus induction on 4.4 g/L of MS at 4 mg/L of 2,4-D. **C:** 42 days of callus induction on 2.2 g/L of MS at 6 mg/L of 2,4-D. **D:** 42 days of callus induction on 4.4 g/L of MS at 6 mg/L of 2,4-D. **E:** 42 days of callus induction on 1.1 g/L of MS at 4 mg/L of 2,4-D. **F:** 42 days of callus induction on 4.4 g/L of MS at 10 mg/L of 2,4-D.

Coleoptile explant on callus induction medium was the highest in callus formation percentage with 100% at 3 mg/L, 4 mg/L, 5 mg/L, and 6 mg/L of 2,4-D for 4.4 g/L of MS media than the others. Besides, callus induction was the lowest at the concentration of 10 mg/L of 2,4-D in 4.4 g/L MS media with 40%. For the score of callus sizes; 4 mg/L and 6 mg/L of 2,4-D in 4,4 g/L MS media led to the largest callus sizes, although 0.5 mg/L of 2,4-D had the smallest. (Table 4.2).

Coleoptile explant had the highest callus formation percentage with 67 % at 5 mg/L of 2,4-D in 2.2 g/L of MS media. However, 0.5 mg/L, 1 mg/L, and 2 mg/L of 2,4-D caused the lowest callus formation percentage with 20%. 3 mg/L, 4 mg/L, 5 mg/L and 6 mg/L of 2,4-D concentrations caused the largest callus sizes. On the other hand, 0.5 mg/L of 2,4-D in 2.2 g/L of MS had to the smallest size. (Table 4.2).

For 1.1 g/L of MS, there were no significant differences for callus formation between the concentration of 2,4-D. However, 5 mg/L 2,4-D had the highest callus formation percentage (60%). The score of callus size shown that 5 mg/L of 2,4-D caused the largest callus sizes. On the other hand, 10 mg/L of 2,4-D in 1.1 g/L of MS resulted in the smallest callus sizes for coleoptile explant (Table 4.2).

Table 4.2. Effect of different concentrations of 2,4-D and MS media on callus formation from coleoptile explant.

Influence of MS media and 2,4-D on coleoptile explant callus induction						
Auxin (2,4-D) (mg/L)	**4.4 g/L MS**		**2.2 g/L MS**		**1.1 g/L MS**	
	Callus percentage[t]	**Callus size**	**Callus percentage[t]**	**Callus size**	**Callus percentage[t]**	**Callus size**
0	0	0	0	0	0	0
0.5	60^{bc}	+	20^{de}	+	47^{bcd}	++
1.0	60^{bc}	++	20^{de}	++	40^{cd}	++
2.0	80^{ab}	++	20^{de}	++	47^{bcd}	++
3.0	100^{a}	+++	33^{cd}	+++	47^{bcd}	+
4.0	100^{a}	++++	47^{bcd}	+++	40^{bcd}	++
5.0	100^{a}	++++	67^{bc}	+++	60^{bc}	+++
6.0	100^{a}	++++	53^{bcd}	+++	53^{bcd}	++
8.0	80^{ab}	+++	47^{bcd}	++	47^{bcd}	++
10.0	40^{cd}	++	47^{bcd}	++	33^{cd}	++

[t] Different letters indicate significantly differences at $p < 0.05$.
+: Tiny, ++: Small, +++: Medium, ++++: Large.

Comparing the callus formation from coleoptile and root explants at different amount of MS media, 4.4 g/L of MS was better than other two concentrations of MS. In the same time, 4.4 g/L of MS media worked better with a low concentration of 2,4-D comparing to the other two MS media concentrations.

4.3 Shoot Induction

In shooting induction from callus structure, 4.4 g/L of MS media supplemented with different doses of TDZ was used (Table 4.3). After cultured callus on shooting media, petri plates were kept in climate room for six weeks. When shoot length grown longer, each sample were transferred alone in the magenta bottle. After six weeks of culture, mean frequency (%) of explants developing shoots and

mean number of shoots per callus were analyzed. The results indicated that the coleoptile explant shooting percentage was between 22% - 67%.

In order to produce indirect shoot regeneration from callus derived from coleoptile explants cultured in MS medium with six different doses of TDZ were used (Table 4.3). Each TDZ hormone treatments were suitable for shoot regeneration. The highest regeneration percentage was observed at 5 mg/L of TDZ with 67%. However, 0.5 mg/L of TDZ had the lowest shoot formation with 22%. 5 mg/L of TDZ had the highest mean number of shoots per callus with 7.0 ± 1.1 although 0.5 mg/L of TDZ had the lowest mean number of shoots per callus with 0.66 ± 0.12. Shoot formation was observed at the doses of 0.5 mg/L, 1 mg/L and 2 mg/L of TDZ; however, shoot did not grow. 3 mg/L and 5 mg/L of TDZ treatments were resulted in a good shoot formation and growth.

Table 4.3. Indirect shoot regeneration from coleoptile explants of *in vitro* grown seedlings of einkorn wheat cultured on MS medium containing different concentrations of TDZ.

Cytokinin (TDZ) (mg/L)	Mean frequency (%) of explants developing shoots[t]	Mean number of shoots per callus[t]
0	0	0
0.5	22^{cb}	0.66 ± 0.12^{c}
1.0	33^{abc}	1.2 ± 0.44^{c}
2.0	33^{abc}	3.3 ± 0.60^{b}
3.0	56^{ab}	5.0 ± 0.58^{b}
5.0	67^{a}	7.0 ± 1.1^{a}

[t] Different letters in the same column indicate significantly differences at $p < 0.05$.

After 12 days, micro shoots were visible on coleoptile callus which cultured on shoot induction media and everyday number of shoots per callus and shoot length were increased (Figure 4.6).

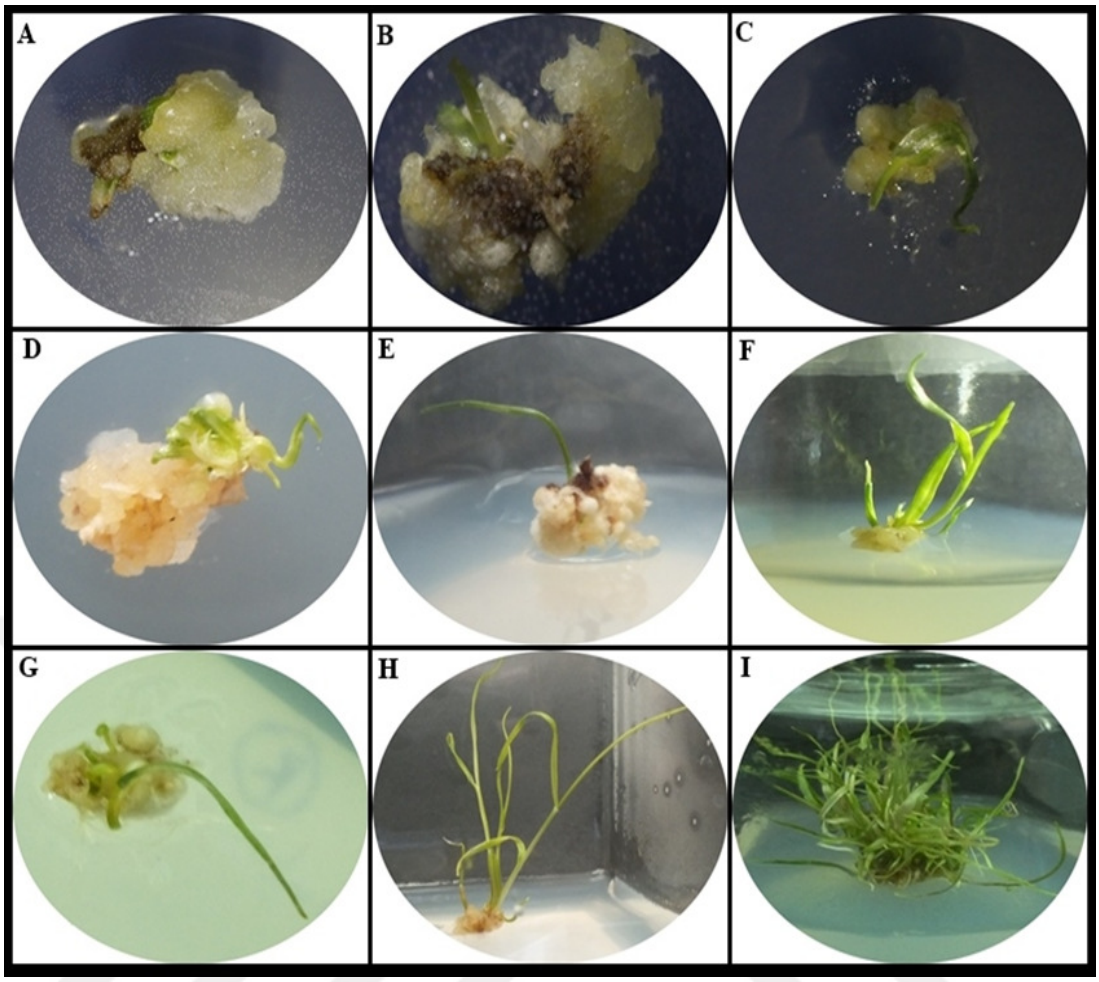

Figure 4.6. The effect of TDZ into 4.4 g/L MS medium on the shoot regeneration from coleoptile explant. **A:** 14 days of shoot regeneration at 2 mg/L of TDZ. **B:** 14 days of shoot regeneration at 3 mg/L of TDZ. **C:** 14 days of shoot regeneration at 5 mg/L of TDZ. **D:** 19 days of shoot regeneration at 2 mg/L of TDZ. **E:** 19 days of shoot regeneration 3 mg/L TDZ. **F:** 19 days of shoot regeneration at 5 mg/L of TDZ. **G:** 27 days of shoot regeneration at 2 mg/L of TDZ. **H:** 42 days of shoot regeneration at 3 mg/L of TDZ. **I:** 42 days of shoot regeneration at 5 mg/L of TDZ.

Callus from root explant shown some green spotting at 0.5 mg/L of TDZ treatment but failed to produce any shoot on all combinations of TZD (Figure 4.7). Even after 42 days, the green spotting did not grow and develop.

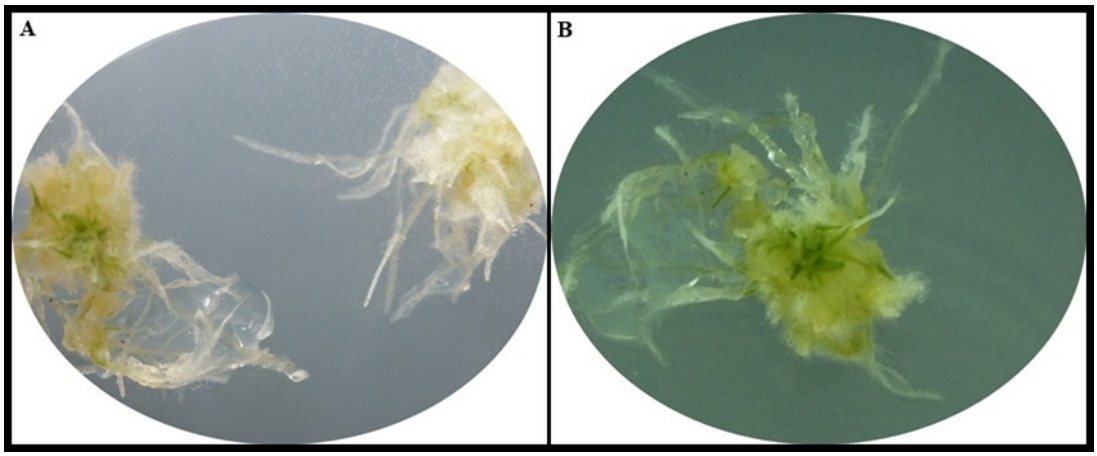

Figure 4.7. The effect of TDZ into 4.4 g/L MS medium on the shoot regeneration from root explant. **A:** 14 days of root callus at 0.5 mg/L TDZ. **B:** 27 days of root callus at 0.5 mg/L of TDZ.

4.4 Root Induction

End of the shoot formation stage, the samples did not generate root. In an attempt to root induction, healthy and well-developed samples with shoots were transferred to root induction media. In order to determine the effect of IAA on rooting of the regenerated shoots from coleoptile explant of einkorn wheat, the seven different doses of IAA were tested (Table 4.4).

For root induction, 4.4 g/L of MS media was supplemented with different doses of IAA. After cultured samples on rooting media, magenta bottles were kept in a climate room for four weeks. Every 15 days, subcultures were made. After four weeks of culture, mean frequency (%) of explants developed root and mean number of roots per shoots data were statistically analyzed.

All IAA treatments were suitable for the root induction. The mean number of roots per shoots range were between 1.7 – 7.7. The best dose for root induction media with root formation percentage, number of roots per rooted shoots, and root length was at 6 mg/L of IAA. The values were 100%, 7.7 ± 0.88 and 4.17± 1.5 respectively. On the other hand, The IAA was failed to affect the length of roots at all doses. The results indicated that mean of longest roots length shown a significant difference at 6 mg/L IAA. (Table 4.4).

Table 4.4. Effect of different concentrations of IAA on rooting of the regenerated shoots from coleoptile explant of einkorn wheat.

Auxin (IAA) (mg/L)	Percentage of shoots that rooted	Mean number of roots per rooted shoots[t]	Mean of longest roots length (cm)[t]
0	0	0	0
1.0	100	1.7 ± 0.33^{de}	$1,3 \pm 0.44^{b}$
2.0	100	4.0 ± 1.15^{bc}	1.6 ± 0.67^{ab}
4.0	100	5.33 ± 0.89^{b}	1.16 ± 0.44^{b}
6.0	100	7.7 ± 0.88^{a}	4.17 ± 1.5^{a}
8.0	100	3.0 ± 0.58^{cd}	2.33 ± 0.89^{ab}
10.0	100	2.3 ± 0.33^{cd}	2.2 ± 0.99^{ab}

[t] Different letters in the same column indicate significantly differences at $p < 0.05$.

After 15 days of culturing, root formation started and become visible. In root induction media, the number of shoots and shoot length were increased (Figure 4.8).

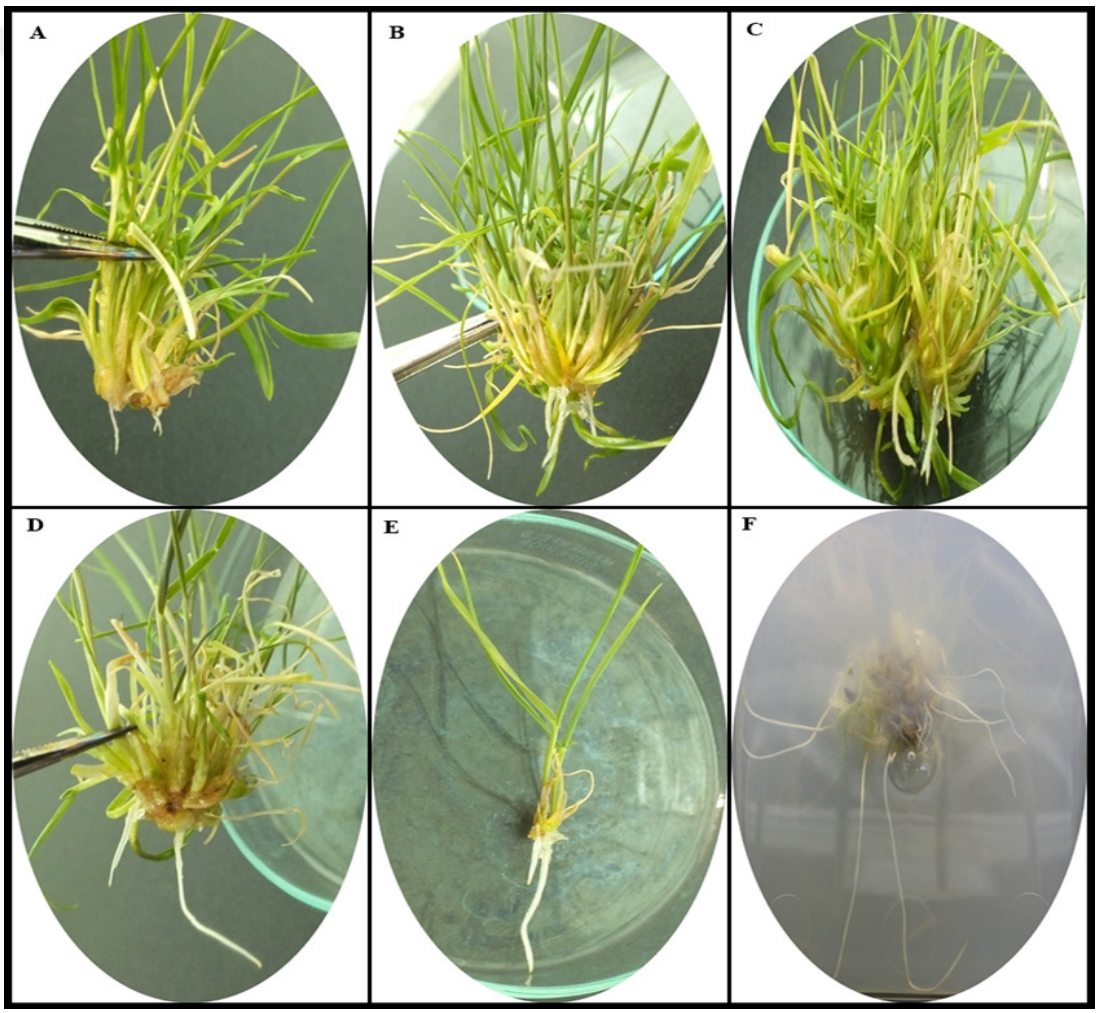

Figure 4.8. The effect of IAA into 4.4 g/L MS media on the root regeneration from coleoptile explant. **A:** 20 days of root regeneration at 1 mg/L of IAA. **B:** 20 days of root regeneration at 4 mg/L of IAA. **C:** 20 days of root regeneration at 8 mg/L of IAA. **D:** 20 days of root regeneration at 10 mg/L of IAA. **E:** 20 days of root regeneration at 2 mg/L of IAA. **F:** 28 days of root regeneration at 6 mg/L of IAA.

4.5 Root and Shoot Growth

After regeneration of shoot and root, plant samples were cultured for 15 days at three different amounts of MS media in order to investigate the response under different MS concentrations of root and shoot growth from coleoptile explant (Table 4.5). There was no significant difference in the mean number of roots between 1.1 g/L and 2.2 g/L of MS. However, 4.4 g/L of MS significantly decreased the mean number of roots when compared to 1.1 g/L of MS. Similarly, there was no significant differences in the mean number of shoots between 1.1 g/L and 2.2 g/L of MS. However, 4.4 g/L of MS reduced in the mean number of shoots in contrast to 1.1 g/L of MS. Mean of the longest root length was obtained in the concentration of 1.1 g/L of MS. On the other hand, 2.2 g/L of MS resulted in the longest shoot. 4.4 g/L of MS significantly reduced the mean of the longest shoot length in comparison to the others.

Table 4.5. Effect of different amounts of MS media on root and shoot growth from coleoptile explant of einkorn wheat.

Amount of MS (g/L)	Mean number of roots[t]	Mean number of shoots[t]	Mean of longest root length (cm)[t]	Mean of longest shoot length (cm)[t]
1.1	13.7 ± 2.9^{a}	12.0 ± 3.8^{a}	8.0 ± 0.50^{a}	17.0 ± 0.50^{b}
2.2	11.3 ± 3.0^{ab}	12.3 ± 0.58^{a}	5.7 ± 0.44^{b}	21.3 ± 3.0^{a}
4.4	8.7 ± 1.15^{b}	5.3 ± 1.5^{b}	4.16 ± 0.44^{b}	12.5 ± 0.76^{c}

[t] Different letters in the same column indicate significantly differences at $p < 0.05$.

During 15 days, number and length of shoot and root were increased compared to the regeneration phase as an observation (Figure 4.9).

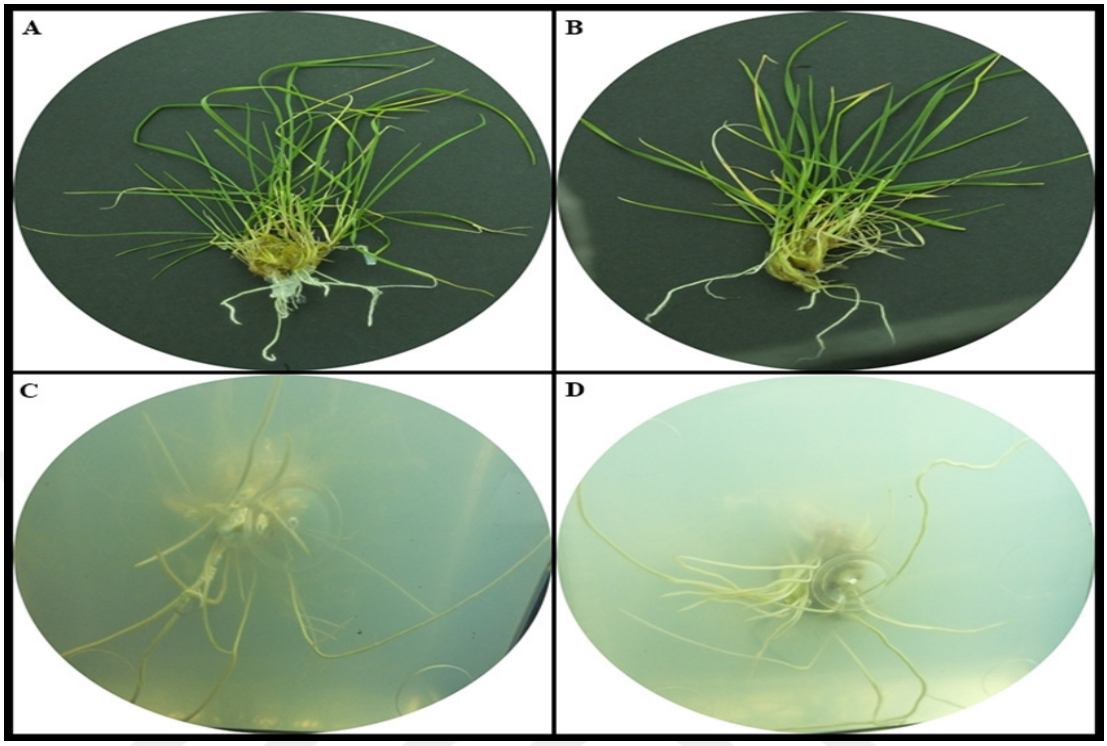

Figure 4.9. The effect of the different amount of MS media on the root and shoot growth from coleoptile explant. **A:** 15 days into 2.2 g/L MS media. **B:** 15 days into 1.1 g/L MS media. **C:** 15 days into 2.2 g/L MS media. **D:** 15 days into 1.1 g/L MS media**.**

4.6 Acclimatization

Well-developed wheat plant samples were transferred to soil for the last stage of *in vitro* propagation of einkorn (*Triticum monococcum* spp. *monococcum*) wheat.

During the acclimatization stage, the number of shoot and length of the shoot were highly increased. After 13 days polythene bags were toked out and einkorn wheat sample was kept in the climate room. After 100 days in the soil, the einkorn wheat headed (Figure 4.10). However, fertilization or grain filling did not happen.

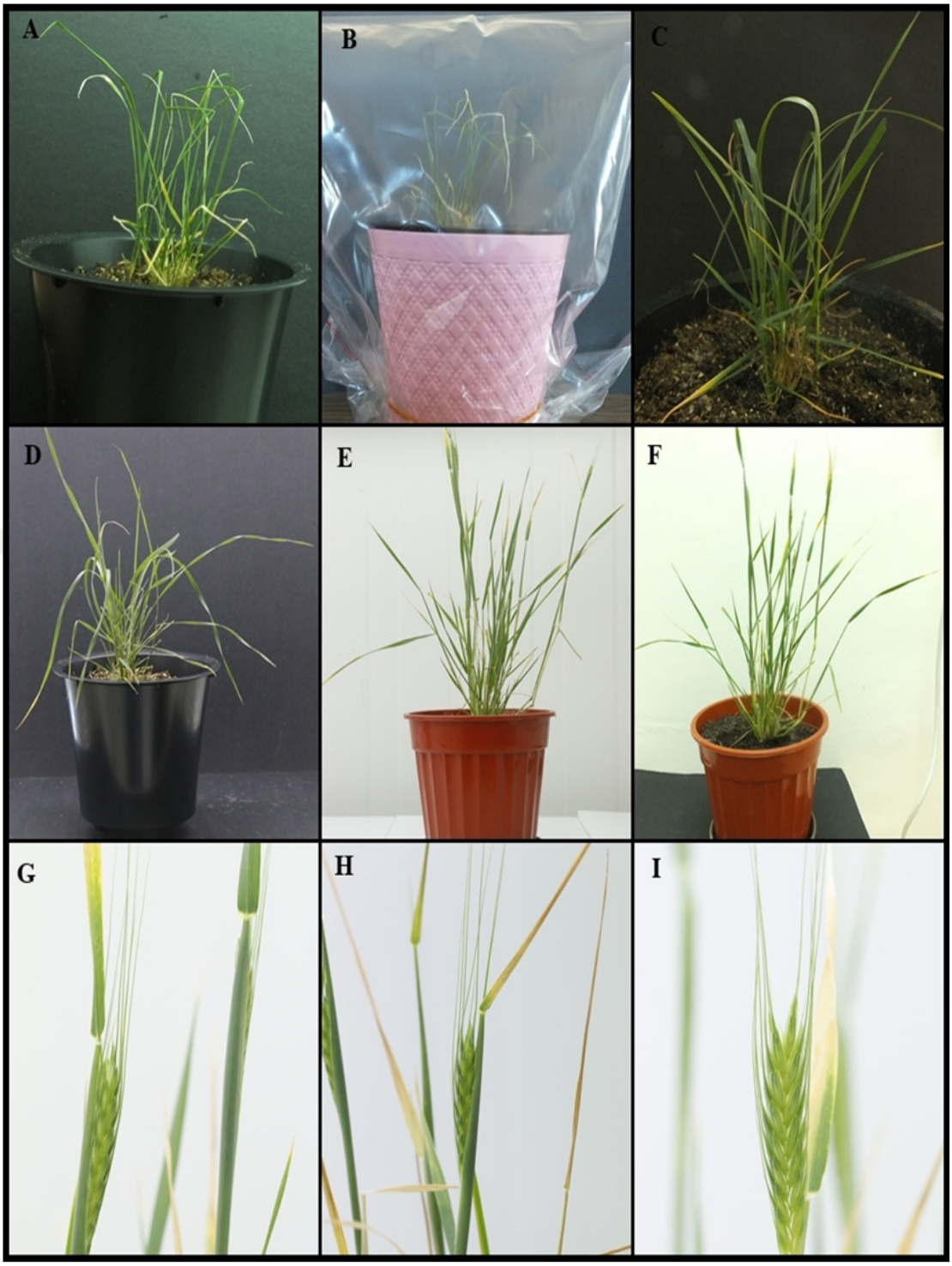

Figure 4.10. Acclimatization stage of einkorn wheat. **A:** First day of regenerated einkorn wheat in the soil. **B:** First day of regenerated einkorn wheat with polythene bags in the soil. **C:** 15 days of regenerated einkorn wheat in the soil. **D:** 30 days of regenerated einkorn wheat in the soil. **E:** 100 days of regenerated einkorn wheat with wheat head in the soil. **F:** 100 days of regenerated einkorn wheat with wheat head in the soil. **G:** 100 days of regenerated einkorn wheat with wheat head in the soil. **H:** 104 days of regenerated.

5. DISCUSSIONS

The main findings of this study were, as stated in the result section, (i) leaf of einkorn wheat did not give callus, only root and coleoptile explant was suitable for callus formation, callus from root was not shoot regenerated while from coleoptile it was regenerated, (ii) the efficiency of hormones in plant tissue culture exerts differences depending on MS concentration, (iii) for callus induction from root and coleoptile segment with considering callus size and percentage, 2,4-D is mainly effective at the concentration of 4 mg/L for root explant and the range between 4-5 mg/L for coleoptile explant in all different dose of MS, (iv) TDZ was effective in indirect shoot regeneration from coleoptile explant at the concentration of 5 mg/L, (v) IAA exerts its best action on the number of roots per shootst in coleoptile explant of einkorn wheats at the concentration 6 mg/L. All dose of IAA was also effective in the formation of root, (vi) as the concentration of MS media increased, the number of roots, shoots and the length of roots decreased, (vii) 2.2 g/L of MS gave rise to the longest shoot length.

As known, *in vitro* propagation is used for vegetative production from different explants types for commercial purposes (Hill and Schaller, 2013). Auxins and cytokinins are needed for cell division, cell enlargement and meristem establishment. The various ratio and combinations of these hormones are important for root and shoot development (Sugimoto et al., 2011).

Organ regeneration in plant tissue culture can be categorized as direct or indirect organogenesis (Sugimoto and Meyerowitz, 2013). Formation of shoot and root directly from explant called as direct organogenesis. Indirect organogenesis includes callus step as an intermediate precursor for shoot and root formation. Indirect organogenesis has three stages which are called competence, determination and, morphological differentiation. In competence stage, explant source enters the dedifferentiation phase. In determination phase, undifferentiated cell structure (callus) decide which part of the plants is formed such as shoot or root according to different combinations of plant growth regulators. Plant growth regulators are

important at the last phase of indirect organogenesis, morphological differentiation is clear and whole plant formation occurs. Organogenesis can be affected by explant type, explant age, explant size and, media composition.

In this study, we focused on to determine an alternative indirect organogenesis protocol using different explant sources, different concentrations of plant growth regulators and MS for einkorn (*Triticum monococcum* ssp. *monococcum*) wheat.

In wheat species, the most common used explant types are immature and mature embryos (Özgen et al., 1996). Both of these explants have advantageous and disadvantageous. For this reason, we choose the root, coleoptile, and leave explants as an alternative explant sources for an efficient indirect organogenesis protocol of einkorn wheat. These three explants can be used throughout the year after germination is completed and these explants can create new opportunities to improve wheat for tissue culture approaches.

There are various types of plant hormones are present and their concentrations and combinations are so important for tissue culture study. Auxin, for example, has an important role on cell division and dedifferentiation (Dudits et al., 1991). Choosing concentrations and type of auxins are highly important because they highly effect on cell division, regeneration, and callus formation in plant. (Barro et al., 1999). In tissue culturing of cereals, 2,4-D, type of auxin, is commonly used as a growth regulator in callus induction media (Naqvi et al., 2002). Generally, 2,4-D is used in the range of 1-3 mg/L for immature and mature embryo culture of cereals (Bi et al., 2007). In this thesis study, we tested the effects of 10 different concentrations of 2,4-D (0.5-10 mg/L) with 3 different concentrations of MS for callus induction.

In this present study, root explant of 5 days seedlings was used for callus formation. Respectively, 4 mg/L of 2,4-D in 4.4 g/L of MS, 8 mg/L of 2,4-D in 2.2 and 1.1 g/L of MS media induced the highest callus formation and callus size. Our study indicated that root explant needed higher 2,4-D concentration with the lower amount (2.2 g/L and 1.1 g/L) of MS for efficient callus formation. 4.4 g/L of MS medium had positive effect on callus size. On the contrary, high amount of 2,4-D

such as 8 and 10 mg/L of 2,4-D in 4.4 g/L of MS and 10 mg/L of 2,4-D for 2.2 g/L and 1.1 g/L of MS caused an adverse for callus induction.

The previous study on root tip segments *Triticum aestivum* L., showed that 5.5 mg/L of 2,4-D was the best hormone concentration for callus formation (Sarker and Biswas, 2002) and this study shown similarities with our study.

It was also shown in the previous study of root explant on rice species indicated that three days old germinated root explant (96.0%) shown better callus formation percentage than five days old root explant (89.3%). 2 mg/L of 2,4-D was also, optimum for callus formation when explants were incubated at 29 ± 1 ºC within eight weeks in the dark (Hoque and Mansfield, 2004). However, in this study, when we used five days old germinated root explants, they gave 67% callus formation at 2 mg/L of 2,4-D which was lower than the study of Hoque and Mansfield (2004). These discrepancies showed that callus formation may be affected by plant species, plant genotype, developmental stage, and culture conditions (Jain, 1997).

In this study, after five days germination of einkorn seed, coleoptile was used as an explant source for callus induction. After 4 – 5 days, callus induction started from cut ends of coleoptile.

We found also similar results on the effects of MS media and 2,4-D concentrations on callus formation percentage of coleoptile explant when compared to root explant. The highest callus formation with 100% was obtained when 4.4 g/L of MS medium with 3, 4, 5 and, 6 mg/L of 2,4-D were used. However, decreasing MS concentration caused increasing 2,4-D concentration for efficient callus formation. 5 mg/L of 2,4-D in 2.2 g/L and 1.1 g/L of MS media had the highest callus formation percentage with 67% and 60%, respectively. According to our results, micro and macro nutrients which are found in MS media were highly effective for callus formation and development from coleoptile explant.

In a previous study, callus and regeneration potential of coleoptile explant was investigated in ten cultivars of *Triticum turgidum* ssp. *durum* (Benkirane et al., 2000). According to that study, callus formation percentage was between 27-32%

and 2,4-D did not significantly affect callus induction. In our study, 2,4-D was effective on callus induction. In another study showed that different genotypes were also effective on callus formation (Mahmood et al., 2012).

We had no embryogenic callus formation in all treatments similarly, the previous study related to *Triticum turgidum* ssp. *durum* (Benkirane et al., 2000).

As we mentioned before, in *in vitro* studies of immature and mature embryo cultures of cereal, 1-3 mg/L of 2,4-D was an efficient concentration for callus formation (Bi et al., 2007). However, in our study, 4.0 mg/L of 2,4-D in 4.4 g/L MS and 8 mg/L of 2,4-D in 2.2 g/L and 1.1 g/L of MS induced the highest callus formation and development from root explant. On the other hand, 3, 4, 5. and, 6 mg/L of 2,4-D in 4.4 g/L MS and 5 mg/L of 2,4-D in 2.2 g/L and 5 mg/L of 2,4-D in 1.1 g/L of MS caused to the highest callus formation induced from coleoptile explant. These differences can be explained by the fact that coleoptile and root explant contain more older cells and differentiated tissues than immature and mature embryos. Due to this reason, higher concentration of auxins may be needed for dedifferentiation of root and coleoptile explants (Mahmood and Razzaq, 2017).

On the other hand, some previous studies related to leaf basal explant regeneration potential and callus formation ability of *Triticum aestivum* L. were reported (Wang and Wei, 2004; Yu et al., 2012). These findings suggested that 4.4 g/L of MS medium was the best for callus formation induced from leaf basal explant as compared to N6 medium and modified L3 medium (Wang and Wei, 2004). According to another study, 2 mg/L of 2,4-D and 1 mg/L of NAA hormone combination was the best for callus formation (87.2%) from leaf basal explant (Yu et al., 2012).

When leaf explant results examined in our study, leaf explant was started to become brownish color on cut end after 4 -5 days on callus induction media. In 10 days, necrosis was started to occur on leaf explant and did not form any callus for all 2,4-D and MS media combinations. Our results of leaf explant in callus induction media has similar result with a previous study (Sarker and Biswas, 2002). In that study, leaf explant did not form callus in all combination of 2,4-D.

In cereal tissue cultures, the fewer formation of plantlets can be increasing by the addition of cytokinin into regeneration media (Schulze, 2007). TDZ is a cytokinin that includes many responses which are similar to natural cytokinin (Guo et al., 2011). TDZ conduces toward to shoot regeneration in many plant species and has an important role in morphogenesis. A previous study was showed that TDZ promoted the regeneration of wheat callus over the other cytokinins (Miroshnichenko et al., 2016). TDZ is well known to be a powerful plant hormone for shoot regeneration (Verma et al., 2011). TDZ alone in media favored plant regeneration more than TDZ combined with auxins as well (Tian et al., 1994). For this reason, in this study we studied 6 different TDZ concentrations with 4.4 g/L of MS media for plant regeneration.

Callus derived from root explant did not shoot regenerate in regeneration media. It just produced some green spot and failed produce shoots. A previous study on *Triticum aestivum* L. was similar result with callus derived from root tip explant (Sarker and Biswas, 2002).

On the other hand, three, five, seven and, nine days old root explants were used in a rice *in vitro* study (Hoque and Mansfield et al., 2004). In that study, embryonic callus derived from root explants were cultured in two different regeneration media; 2 mg/L of BAP, 1 mg/L of NAA and 1 mg/L of KIN. The other medium was 2 mg/L of BAP with 0.5 mg/L of NAA. Their result suggested that three days old root segment regenerated the best green plants. However, in the study here, callus derived from root explant did not shoot regenerate. The explanation is that the plant species, hormones and explant ages used in these two studies were different and it most likely altered regeneration stage.

Well-developed callus from coleoptile explant after exposed to TDZ induced shoot formation as reported by other studies (Varshney et al., 1997; Benkirane et al., 2000). Here different percentage of regeneration and mean number of shoots per callus were observed under different concentrations on TDZ treatment at 4.4 g/L of MS. The concentration of TDZ significantly influenced the regeneration of callus derived from coleoptile explant. Low concentrations of TDZ such as 0.5 mg/L, 1 mg/L and, 2 mg/L were less efficient on regeneration and 5 mg/L of TDZ had the

highest regeneration percentage. However, a previous study, above mentioned, on common winter wheat (*Triticum aestivum* L.), 0.75 mg/L of TDZ was the optimal concentration for shoot formation from callus of immature embryos (Benlioğlu and Birsin, 2017). Using two different explants types and wheat species are supposed to explain these differences.

IAA is commonly detected natural auxin and it is effective on root formation for *in vitro* studies. In some previous studies IAA was used between 0.5 mg/L and 1 mg/L (Kothari and Varshney, 1998; Kopertekh and Stribnaya, 2003) for wheat root initiation.

Regenerated shoots from coleoptile explant did not form any root structure in regeneration media even after 42 days. For root formation IAA hormone was used at six different concentrations to determine the best IAA concentration for einkorn wheat. All concentrations of IAA worked well for root induction and 6 mg/L of IAA was found the best for root formation mean number of roots per shoots and mean of the longest root length. Increasing the concentration of IAA in the media decreased the number of roots per shoots.

Before the acclimatization of einkorn wheat, different amounts of MS (4.4, 2.2, and 1.1 gr/L) were investigated on shoot and root growth. The previous study on *Typhonium flagelliforme* shown that 1.1 gr/L of MS media induced maximum shoot and root number when compared to 4.4 g/L of MS and 2.2 g/L of MS (Rezali et al., 2017). Similar, results were presented in this thesis study for root growth and development. On the other hand, 2.2 g/L of MS media had the best shoot growth and development.

6. CONCLUSION AND RECOMMENDATIONS

In this study, *in vitro* einkorn wheat regeneration protocol by using different types of explants, including root, coleoptile and leaf was investigated. Indirect organogenesis, except leaf and root explant, was induced at all concentrations of 2,4-D, TDZ and IAA used. Furthermore, callus from coleoptile explant was suitable for the formation of shoot and root under regenerations media. The callus and regeneration protocol of einkorn wheat used in this study is expected to new opportunities for future studies in the wheat improvement programs. Such studies are supposed to be great importance in order to solve any problem that wheat breeders and agricultural scientists face.

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8. CURRICULUM VITAE

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List of Publications **:**

1. **Örgeç, M.,** Karakaş, FP., Şahin, G., Ağıl, F., Zencirci, N., (2018). "Einkorn (*Triticum monococcum* ssp. *monococcum*) *in vitro* propagation sterilization protocol", International Journal of Secondary Metabolite, 5 (2), 67-74. DOI: 10.21448/ijsm.399094
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1. **Örgeç, M.,** Şahin, G., Karakaş PF., Zencirci, N., "Effect of auxin and citokinin combinations on Regeneration of hulled einkorn (*Triticum monococcum* ssp. *monococcum*) wheat", Uluslararası Tıbbi ve Aromatik Bitkiler Kongresi - "Tabii ve Sağlıklı Hayat". Konya/TÜRKİYE, 9 -12 Mayıs 2017. (Poster Presentation)
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