# OPTIMIZATION OF MATURE EMBRYO BASED REGENERATION AND GENETIC TRANSFORMATION OF TURKISH WHEAT CULTIVARS

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**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.**

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## ABSTRACT

OPTIMIZATION OF MATURE EMBRYO BASED REGENERATION AND GENETIC TRANSFORMATION OF TURKISH WHEAT CULTIVARS

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The objective of this study was to optimize tissue culture, transformation and regeneration parameters of mature embryo based culture of *Triticum durum* cv. Mirzabey 2000 and *Triticum aestivum* cv. Yüreğir 89. The effects of auxin type of hormone at different concentrations and dark incubation periods on regeneration capacity were evaluated. Two different hormone types 2,4- dichlorophenoxyacetic acid and picloram were used at three different concentrations 2, 4 and 8 mg/l. Mature embryo derived calli were incubated in 6 different induction media at dark for 4 and 6 weeks for initiation of primary callus induction. After dark incubation periods, average callus fresh weight and primary callus induction rate were determined. The primary callus induction rates for 4 weeks and 6 weeks old dark adapted Mirzabey calli incubated was found to be 91 % and 93.25 % respectively. Yüreğir primary callus induction rate was 92.5 % for 6 weeks old calli in 6W2D medium and 86.75 % for 4 weeks old calli in 4W8P medium. The primary calli were transferred to embryogenic callus induction medium. The embryogenic callus formation was 94.88

in 6W2D medium for Mirzabey cultivar. The necrosis was observed at high concentration of 2,4-D for both of cultivars. After embryogenic callus induction, embryogenic calli were transferred into hormone free regeneration medium. The maximum regeneration rate (62.31 %) and culture efficiency (44.13 %) were observed in 4W2D medium for Mirzabey. However, the low regeneration rate was observed for Yüreğir (5 %) in 6W2D medium.

The transformation studies were performed by using Obitek Biolab Gene Transfer System. The old and the modified loading units were used for optimization of bombardment pressure and distance for mature embryo based calli transformation. After bombardment of pAHC25 coated gold particles, histochemical GUS assay was performed and blue spots were counted. The transformation efficiency increased to 0.65 fold for 30 bar bombardment pressure and 5.5 fold for 35 bar bombardment by the modified loading unit. The modified loading unit could be used for further transformation studies.

Key words: Wheat, mature embryo, regeneration, auxin concentration, particle bombardment

# ÖZ

TÜRK BUĞDAY ÇEŞİTLERİNİN OLGUNLAŞMIŞ EMBRİYO KAYNAKLI REJENERASYONUNUN VE GENETİK TRANSFORMASYONUNUN OPTİMİZE EDİLMESİ

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Bu çalışmanın amacı Mirzabey 2000 (*Triticum durum*) ve Yüreğir 89 (*Triticum aestivum)* buğday çeşitlerinin olgunlaşmış embriyo kaynaklı doku kültürü, transformasyon ve rejenerasyon parametrelerinin optimize edilmesidir. Farklı konsantrasyonlarda ki oksin hormonlarının ve karanlık inkübasyon periyotlarının rejenerasyon kapasitesine etkisi değerlendirilmiştir. İki farklı hormon tipi 2,4-diklorofenoksiasetik asit ve pikloram üç farklı konsantrasyonda 2, 4 ve 8 mg/l kullanılmıştır. Olgunlaşmış embriyo kaynaklı kalluslar birincil kallus oluşumu için 6 farklı indüksiyon besiyerinde 4 ve 6 hafta karanlıkta inkübe edilmişlerdir. Karanlık periyod sonrasında, ortalama taze kallus ağırlığı ve birincil kallus indüksiyon oranı belirlenmiştir. Karanlıkta 4 ve 6 hafta bekletilen Mirzabey kallusları için birincil kallus indüksiyon oranı % 91 ve % 93.25 olarak bulunmuştur. Yüreğir için birincil kallus indüksiyon oranı 6W2D besiyeri için % 92.5 ve 4W8P besiyeri için % 86.75 olarak gözlemlenmiştir. Birincil kalluslar embriyojenik kallus oluşturma besiyerine

taşınmışlardır. Embriyojenik kallus oluşumu Mirzabey çeşidi için 6W2D besiyerinde % 94.88 dir. Her iki çeşit için 2,4-D nin yüksek konsantrasyonlarında nekrosis gözlemlenmiştir. Embriyojenik kallus oluşumundan sonra kalluslar hormon içermeyen rejenerasyon besiyerine alınmışlardır. Mirzabey çeşidi için en yüksek rejenerasyon oranı (%62.31) ve kültür verimliliği (% 44.31) 4W2D besiyerinde gözlemlenmiştir. Fakat, Yüreğir çeşidi için rejenerasyon oranı (% 5) oldukça düşük olarak 6W2D besiyerinde gözlemlenmiştir.

Transformasyon çalışmları Obitek Biolab Gen Transfer Sistemi kullanılarak yapılmıştır. Olgunlaşmış embriyo kaynaklı kalluslar için eski ve modifiye edilmiş yükleme üniteleri kullanılarak bombardıman basıncı ve mesafesi optimize edilmiştir. pAHC25 kaplı altın mikro parçacıkların bombardımanı sonrası histokimyasal GUS deneyi yapılmış ve mavi noktalar sayılmıştır. Modifiye edilmiş sistem kullanılarak tranformasyon verimliliği 30 bar bombardıman basıncı için 0.65 kat ve 35 bar için 5.5 kat artmıştır. Modifiye edilmiş yükleme ünitesi ileri ki transformasyon çalışmalarda kullanılabilir.

Anahtar kelimeler: Buğday, olgun embriyo, rejenerasyon, oksin konsantrasyonu, partikül bombardımanı

To My Wife and My Parents

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| | |
|---|---|
| 2,4-D | 2,4-dichlorophenoxyacetic acid |
| $AgNO_3$ | Silver nitrate |
| ANOVA | Analysis of variance |
| BAP | 6-Benzylaminopurine |
| *bar* | Bialaphos resistance gene |
| bp | Base pair |
| $CuSO_4$ | Cupper sulphate |
| cv | Cultivated variety |
| DNA | Deoxyribonucleic acid |
| GUS | β-glucuronidase |
| IAA | Indole acetic acid |
| LB | Luria broth |
| MS | Murashige-Skoog basal salt medium |
| NAA | 1-Naphthaleneacetic acid |
| NaCl | Sodium chloride |
| NaOCl | Sodium hypo choloride |
| NaOH | Sodium hydroxide |
| *nptII* | Neomycin phosphotransferase II |
| *pat* | Phosphinotricin acetyl transferase |
| PCR | Polymer Chain Reaction |
| PPT | Phosphinotricin |
| RNA | Ribonucleic acid |
| RT-PCR | Real Time Polymer Chain Reaction |
| spp. | Species |

# CHAPTER 1

# INTRODUCTION

## 1.1.The Wheat Plant

Wheat is one of the most important crop plant produced, traded and consumed in the world. It is thought that the cultivation of wheat reaches far back into history. Wheat has been a major food source in the human diet for 8000 years in Europe, West Asia and North Africa. According to Food and Agriculture Organization of United Nations, wheat was grown over 200 million hectares (nearly 17 % of the world's cultivable land) and produced nearly 690 million tons in 2008. From 8000 years ago to today, wheat continues its important role as a food source.

### 1.1.1. Characteristics of Wheat

Wheat is an annual and monocotyledon plant from Gramineae (Poacea) family and *Triticum* genus. The plant is composed of a root and shoot system. The seminal roots and the nodal roots are parts of root system. Phytomers having a node, a leaf, an elongated inter node and a bud in the axil of the leaf make up shoot system. The sheath which envelops the subtending leaf, and a lamina (blade) are parts of a leaf. Tillers called lateral branches originate from the basal leaves axils of the wheat plant. While some tillers produce an ear at anthesis, others die (Kirby, 2002).

Wheat cultivars may have winter or spring growth habit. While winter cultivars are planted in the autumn, spring wheat cultivars are planted in the spring. Winter wheat

cultivars need a vernalization period to initiate reproductive development and floral primordia (Setter and Carlton, 2000). Vernalization is a period having near or slightly below freezing temperatures and length of daylight. Spring wheat cultivars do not need to vernalization period to alter from vegetative growth to reproductive growth (Cook *et al*., 1993).

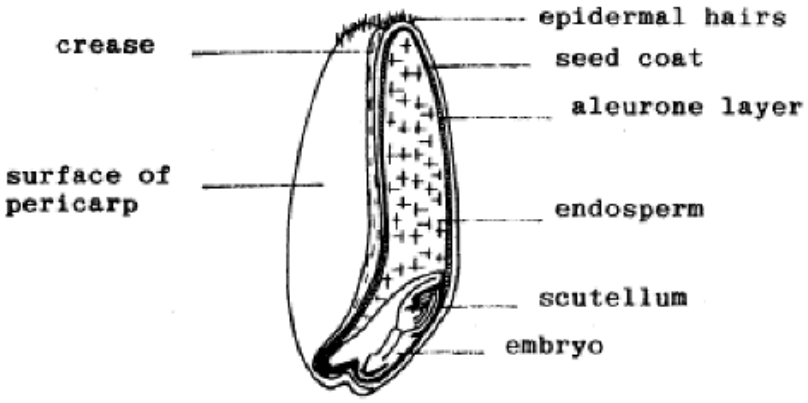


Figure 1. 1. Structure of a wheat kernel
(http://www.regional.org.au/au/roc/1988/roc198823-1.gif, 04.08.2010)

The seed, grain or kernel of wheat (botanically, caryopsis) is a dry indehiscent fruit. Seed size is nearly 4-8 mm long, depending on growth condition and the variety. The dorsal side of seed (with respect to the spikelet axis) is smoothly rounded, while ventral side has the deep crease. The embryo or germ is located at the point of attachment of the spikelet axis, and the distal end has a brush of fine hairs. The scutellum, the plumula (shoot) and the radical (primary root) are parts of embryo. The scutellum is the region that secrets some of the enzymes included in germination and absorbs the soluble sugars from the breakdown of starch in the endosperm. The plumula, which forms the shoot when the seed germinates, has a stem attached to it to the coleoptile, which functions as a protective sheath. The aleurone layer or metabolically active cell layers surround the endosperm, the seed coat or testa and the fruit coat or pericarp.

### 1.1.2. Geographic Origin and Classification

Wheat evolved from wild grasses found growing in the Eastern Mediterranean and the Near East and Middle East areas and in places where other similar cereal crops such as barley and rye possibly developed (Bozzini, 1988). Wheat is a member of Angiosperm class, the monocot sub-class and grass family. Within grass family, wheat is a member of the tribe Triticeae and genus *Triticum* (Cook *et al*., 1993). Different wheat species are given in Table 1.1.

The Fertile Crescent is considered the birth place of cultivated wheat nearly 8000 to 10000 years ago according to archaeological and botanical evidence. Pure stands of wild diploid einkorn and wild tetraploid emmer are found there and may have been harvested and cultivated such as. Diploid einkorn types of wheat are the earliest and the most primitive, while the hexaploids including the bread wheat, *Triticum aestivum*, constitute the most recent and latest step in the evolution of the wheat complex (Patnaik and Khurana, 2001). It is taught that the wild grass *Aegilops speltoides* and *Triticum monococcum* being wild diploid wheat was the first hybridization event millions of years ago, Figure 1.2.

**Table 1. 1.** Classification of the wheat species *Triticum* L (based on Van Slageren, 1994).

| Species | Subspecies | Status | Chromosome number | Genome |
|---|---|---|---|---|
| *T.monococcum* | *aegilopoides* | Wild | 2n=14 | AA |
| | *monococcum* | Cultivated | | |
| *T.urartu* | | Wild | | AABB |
| *T.turgidum* | *cartlicum* | Cultivated | 2n=28 | AABB |
| | *dicoccoides* | Wild | | |
| | *dicoccum* | Cultivated | | |
| | *durum* | Cultivated | | |
| | *turgidum* | Cultivated | | |
| | *paleocolchicum* | Archaelogical | | |
| | *polonicum* | Cultivated | | |
| *T.timopheevii* | *armeniacum* | Wild | 2n=28 | AAGG |
| | *timopheevii* | Cultivated | | |
| *T.aestevum* | *spelta* | Cultivated | 2n=42 | AABBDD |
| | *macha* | Cultivated | | |
| | *aestivum* | Cultivated | | |
| | *compactum* | Cultivated | | |
| | *sphaerococcum* | Cultivated | | |

The result of this hybridization was tetraploid emmer wheat, *Triticum dicoccum*. Domestication of emmer wheat caused to the evolution of the durum wheat (Patnaik and Khurana, 2001). After durum wheat hybridization, hexaploid bread wheat evolved from hybridization between *Triticum turgidum var. durum* (2n=28, AABB) and *Aegilops tauschii* being wild goat grass about 8000 years ago. Bread wheat is thus an allohexaploid, containing three dinstinct but genetically homologous copies each of three originally independent haploid genomes, the A, B and D (Gill and Gill, 1994).

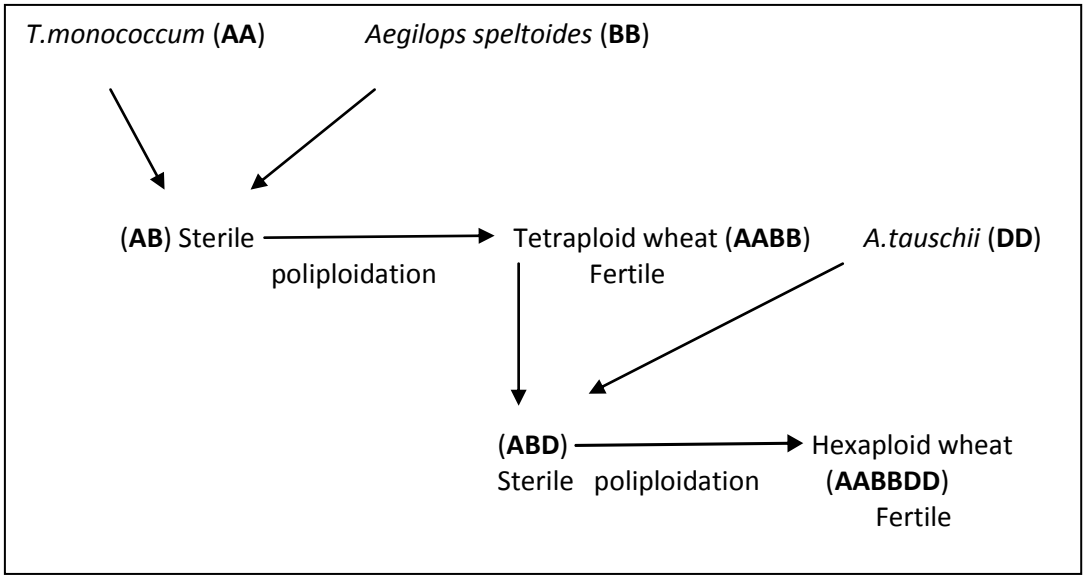


Figure 1. 2. Evolution of wheat

10 different *Triticum* species, namely *T. boeoticum*, *T. monococcum*, *T. timopheevii*, *T. dicoccoides*, *T. dicoccum*, *T. durum*, *T. turgidum*, *T. polonicum*, *T. carthlicum*, *T. aestivum* are grow naturally in Turkey. Two most commonly cultivated wheat cultivars are *T. durum* (2n=48) and *T. aestivum* (2n=42) in Turkey (Tan, 1985).

### 1.1.3. Genetic and cytogenetic characteristics of wheat

The basic chromosome number of wheat species is seven. Thus, diploid wheat species have 14 chromosomes, the tetraploid emmer and modern durum wheat species have 28 chromosomes and the common hexaploid wheat species have 42 chromosomes (Cook *et al*., 1993).

Tetraploid wheat species arose as the consequence of rare but natural crosses between two diploid wheat species. Through natural hybridization, one diploid species combined its set of chromosomes with different set of chromosomes of another diploid species by a process known as amphidiploidy. The genomes of the different wild diploid species have been labeled by cytologists for scientific purpose as AA, BB, CC, DD. Hexaploid wheat species arose by the same process: a diploid of genome DD combined with a tetraploid of genome AABB to produce a hexaploid hybrid of genome AABBDD (Cook *et al*., 1993).

*Triticum durum*, durum wheat genome size is nearly 10 billion base pairs. This genetic code is located into 28 chromosomes being diploid AABB. The structure of A- and B- genome chromosomes of durum wheat is essentially identical to the corresponding homologues of bread wheat (Gill and Friebe, 2002). *Triticum aestivum*, bread wheat has a genome size of 16 billion base pairs of DNA organized into 21 pairs of chromosomes, seven pairs belonging to each of the genomes A, B, and D (Sears, 1954; Okamoto, 1962; Gill and Friebe, 2002).

### 1.1.4. Nutritional profile of wheat

In many countries, wheat is the most important crop plant of human diet. Wheat based foods is considered the major source of energy, protein and vitamins and minerals. Wheat based foods are consumed as two-thirds or more of the daily caloric intake by some population groups (Ranhotra, 1994).

The wheat kernel composition varies widely. For example, usual range of protein is from 8% to 15%, however 7% or as high as 24% can occur. While 26% protein can be detected in embryo, 24% protein can be detected in aleurone. Gluten, a water insoluble protein fraction, can be isolated from endosperm. Gluten is especially important for the leavening in bread making (Inglett, 1974).

When protein content of wheat is considered, lysine is the most deficient amino acid in wheat. Wheat has low rate fat and the fat that is present is high in unsaturated fatty acids (Rhantora, 1994). Triglycerides, phospholipids and glycolipids, fatty acids, sterols, monoglycerides and diglycerides are present in whole wheat as well as endosperm lipids (Inglett, 1974). Tocopherols are another lipid class present in wheat. Wheat germ is an abundant source of α-tocopherol known as vitamin E (Inglett, 1974).

Wheat flour is a good source of complex carbohydrate. Energy is stored in the starch form in cereal grains. Wheat includes starch between 60% and 75% of total dry weight of grain. Starch occur in seed in the form of granules (Sramkova et.al., 2009) Whole wheat flour and its bran fraction are a good source of fiber, particularly water insoluble fiber. Wheat has also some vitamins and minerals (Rhantora, 1994).

### 1.1.5 Types of wheat and their uses

Wheat may be classified according to protein content, hardness, grain color (red or white) and growth habit. Wheat cultivars are divided into hard red spring wheat, hard red winter wheat, soft white wheat, hard white wheat, soft red winter wheat and durum wheat in The United States and Canada (Cook et al., 1993). If wheat have high amount of protein (13-16%), they can be used for bread making. Wheat having low protein content (8-11%) can be used for pastries, cookies, crackers, flat breads and oriental noodles (Cook et al., 1993).

Table 1. 2. Types and utilizations of wheat
(Adopted from http://www.texaswheat.org/images/E0161001/EDU_wheat_types.pdf, 05.08.2010)

| **Types of wheat** | **Utilization** |
|---|---|
| Hard Red Winter Wheat | With a wide range of protein content, good milling and baking characteristics, it is used to produce bread, rolls and all-purpose flour. |
| Hard Red Spring Wheat | This wheat contains the highest percentage of protein, making it excellent bread wheat with superior milling and baking characteristics. |
| Soft Red Winter Wheat | Has a relatively low percentage of protein. It is used for flat breads, cakes, pastries and crackers. |
| Hard White Wheat | This wheat has a milder, sweeter flavor, equal fiber and similar milling and baking properties. Used mainly in yeast breads, hard rolls, bulgur, tortillas and oriental noodles. |
| Soft White Wheat | High yielding, but with low protein, this wheat is used to produce flour for baking cakes, crackers, cookies, pastries, quick breads, muffins and snack foods. |
| Durum Wheat | The hardest of all wheat and used to make semolina flour for pasta, macaroni, spaghetti and similar products. |

### 1.1.6. Wheat production

More than 120 countries in the world produce wheat and this is one-fifth of the world's calorie needs (Donald et.al., 2005). According to United Nations food and Agricultural Organization (FAO) statistics in 2008, China, India, United States of America, Russian Federation and France are the top five wheat producers. According to FAO statistics wheat production was 690 million tons in 2008. China produced nearly 112.5 million tons (16.3% of total production) of wheat. Turkey produced 17.8 million tons of wheat (2.6% of total production). 7.6 million decare area was harvested and yield was 234 kg/decare. While Turkey rank was ninth in 2004, Turkey drawn back to tenth in 2008. The top wheat producer countries from 2004 to 2008 are given in table 1.3.

Table 1. 3. Top wheat producer countries (2004-2008).
(http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567#ancor,05.08.2010)

| **Country** | **2004** | **2005** | **2006** | **2007** | **2008** |
|---|---|---|---|---|---|
| China | 92 | 97.5 | 108.5 | 109.3 | 112.5 |
| India | 72.2 | 68.6 | 69.4 | 75.8 | 78.6 |
| United States | 58.7 | 57.3 | 49.5 | 55.8 | 68 |
| Russia | 45.4 | 47.7 | 45 | 49.4 | 63.8 |
| France | 39.7 | 36.9 | 35.4 | 32.8 | 39 |
| Canada | 24.8 | 25.8 | 25.3 | 20.1 | 28.6 |
| Germany | 25.4 | 23.7 | 22.4 | 20.8 | 26 |
| Australia | 21.9 | 25.2 | 10.8 | 13 | 21.4 |
| Pakistan | 19.5 | 21.6 | 21.3 | 23.3 | 21 |
| Turkey | 21 | 21.5 | 20 | 17.2 | 17.8 |
| **World Total** | **632.7** | **626.9** | **605.1** | **611.1** | **690** |

In terms of million tons.

## 1.2.Improvement of wheat

Wheat is the main food source of 40% of world's population. Wheat provides 500kcal of energy per capita per day in the two most populous countries in the world, China and India and over 1400kcal per capita per day in Iran and Turkey (Dixon et.al, 2009). While 16% of total dietary calories come from wheat in developing countries, this rate is 26% in developed countries (Dixon et.al, 2009). It is forecast that The World's population will be 12 billion in 2050 because of addition of two hundred people in every minute to human population. It is also predicted that annually wheat production will be 760 million tons in 2020, 813 million tons in 2030 and 900 million tons in 2050 (Rosegrant et.al, 2001). The regular increase in wheat production continues due to increases in both area and yield. Production area continuously expanded for many decades. Scientists have been studying to increase wheat yield using conventional breeding and biotechnological techniques.

### 1.2.1. Conventional breeding

Scientific approaches to crop improvement go back rediscovery of Mendel's law at the beginning of this century. After Mendel's law rediscovery, new technologies have been investigated to increase wheat traits such as increase grain yield caused by biotic and abiotic stress and disease resistance originated by insects and microorganisms (Pingali and Rajaram, 1999).

Processes of crossing, back crossing and selection are used by breeders as conventional breeding techniques. Pure lines, multilines and hybrids can be developed using these breeding techniques. Firstly, pure lines are produced by cross-breeding. After that, genetically uniform lines are selected. Cross of more than one line is called multilines. Cytoplasmic male-sterile method and chemical hybridization agent method can be used to produce hybrids. The most common ones are pure line crossbred cultivars (Cook et al., 1993)

The tribe *Triticeae* having over 300 species including wheat, rye and barley gives chance to breeders as a germplasm source to improve new cultivars (Patnaik and Khurana, 2001). Increase pest and disease resistance, drought and salt tolerance, better grain quality and value added traits are enormous possibilities for wheat improvement. Wheat gene pool and its relatives can be a clue for some of these improvements (Janakiraman et al, 2002). However, much of the world wheat germplasm traits are not suitable for modern processing of wheat grain for some foods products. If wheat germplasm can be used to improve new cultivars, many generations of backcrosses and selection must be done (Cook et al., 1993). Although, breeders have used conventional method to improve the yield of wheat for a long time, but yield does not significantly increase (Sahrawat et al, 2003).

### 1.2.2. Wheat Biotechnology

Biotechnological approaches are considered the latest tools for agricultural researches. Besides plant breeding applications, biotechnology focuses on to development of novel methods for genetically alteration and control of plant development, performance and products. The delivery, integration and expression of defined genes into plant cells are parts of plant biotechnology. While conventional breeders use domestic crop cultivars and its relative genus as a gene source to improve new cultivars, biotechnologists can use defined genes from any organism. After exogenous genes are introduced to as a heritable character to wheat by using biotechnological techniques, the availability of desired genes is important for development of wheat (Patnaik and Khurana, 2001).

Conventional wheat breeders challenge to increase grain yield and to get minimum crop loss because of bad environmental or biotic conditions have been considerably important up to now. After the middle of this century, conventional breeding programs increased two times of the world wheat production. This increase caused green revolution. Afterwards, studies moved the decrease of yield variability because of biotic and abiotic stress and benefit from input-use efficiency (Pingali and Rajarm,

1999). Researchers have developed two possible solution against changing global food policy: firstly decreasing farm input spending to overcome biotic and abiotic stress factors improving resistant wheat cultivars against these changing conditions and secondly, increasing product quality such as developing nutritional profile, appearance of end product and processing or storage characters . Foreign genes introduced to a new cultivar encode agronomically important traits. As a result, increasing the yield, quality characters, resistance to biotic stress and tolerance to abiotic stress are mainly study areas to be focused on by researchers (Patnaik and Khurana, 2001).

Transformation, cell and tissue culture, genome mapping's and molecular markers, double haploids, gene isolation, sequencing and bioinformatics are wheat biotechnology research approaches. Tissue culture and transformation studies are two important points in wheat biotechnology. There are three parameters about these studies. The suitable and highly efficient regeneration system improvement is first step of wheat biotechnology research. Secondly, gene delivery technique, reliable and highly efficient, should be developed to introduce the desirable traits to the wheat plants. Finally, a good working screening and selection methods should be used to achieve healthy and usable transformants. Scientists continue to study in order to improve new wheat cultivars having desired new traits by using both regeneration and transformation studies.

#### 1.2.2.1. Plant tissue culture

Production of a large number of regenerable cells is the main aim of the plant tissue culture studies. After development of regeneration system, transformation studies should be done to deliver of desirable genes into plants. It is considered that regeneration studies is the most difficult part of tissue culture (Slater et al., 2008).

#### 1.2.2.1.1. General characteristics of plant cell culture

##### 1.2.2.1.1.1.Plasticity and totipotency

Plasticity and totipotency are two important points to understand plant cell culture and regeneration. Plant growth and development processes have a good adaptation to different environment condition because of their sessile nature and long life. This plasticity gives chance to the plants to change their metabolisms, growth and development to best suit their environment. Plasticity provides one type of tissue or organ to be initiated from another type. Any tissue of the plant can be used to start cell division because of this plasticity to regenerate lost organs or suffer from different developmental pathways in response to particular stimuli. When plant cells and tissues are cultured in vitro they generally have a very high degree of plasticity. Using this capacity, whole plants can be regenerated. This regeneration of whole organisms depends upon the concept that all plant cells can, given the correct stimuli, express the parent plant's total genetic potential. This maintenance of genetic potential is called 'totipotency'. Plant cell culture and regeneration do, in fact, provide the most compelling evidence for totipotency. In practical terms though, identification of the culture conditions and stimuli provided to exhibit this totipotency can be extremely difficult and it is still a largely empirical process (Slater et al., 2008).

#### 1.2.2.1.1.2. Culture environment

The growth medium and the external environment are very important components of *in vitro* cell culture studies. The growth medium includes all the essential mineral ions required for growth and development. Macronutrients (or macroelements) such as nitrogen, phosphorus, potassium, magnesium, calcium and sulphur, micronutrients (or microelements) such as manganese, iodine, copper, cobalt, boron, molybdenum, iron and zinc, and iron source as iron sulphate can be categorized essential elements in vitro cell culture. Some amino acids (glycine, arginine, asparagine, aspartic acid,

alanine, glutamic acid, glutamine and proline and vitamins (thiamine and myoinositol) can be used as additive organic molecules in many cases. Sucrose is the most commonly added to medium as a carbon source. Glucose, maltose, galactose and sorbitol can also be used as carbon source. One other vital component that must also be supplied is water, the principal biological solvent. Liquid or solidified using agar, plant agar and phytagel media can be used in cell culture. Physical factors, such as temperature, pH, the gaseous environment, light (intensity and duration) and osmotic pressure, also have to be maintained within acceptable limits (Slater et al., 2008).

#### 1.2.2.1.1.3. Plant growth regulators

Plant growth regulators, plant hormones or their synthetic analogues, are used to directly manipulate the development of the plant cells in the cell culture. Auxins, cytokinins, gibberellins, absisic acid and ethylene are plant growth regulators used in plant cell culture. Cell division and cell growth are promoted by using auxins. The most commonly used auxin is 2,4-Dichlorophenoxyacetic acid (2,4-D) (Slater et al., 2008).

Cytokinins such as zeatin and its synthetic analogues kinetin and benzylaminopurine (BAP) promote cell division. Absisic acid (ABA) prevents cell division. It is most commonly used in plant tissue culture to promote distinct developmental pathways such as somatic embryogenesis. Ethylene normally controls fruit ripening. It can be a problem for plant tissue culture due to inhibit growth and development of culture. Plant growth regulators have been used in plant tissue culture since 1950s. However, it is difficult to predict their effects on account of the great differences in culture response between species and cultivars. Most wide used growth regulators are auxins and cytokinins. A high auxin to cytokinin ratio generally promotes root formation, whereas a high cytokinin to auxin ratio promotes shoot formation. An intermediate ratio promotes callus production (Slater et al., 2008).

Table 1. 4. Commonly used auxins in cell culture (Slater et al., 2008).

| Abbreviation/name | Chemical name |
|---|---|
| 2,4-D | 2,4-dichlorophenoxyacetic acid |
| 2,4,5-T | 2,4,5-trichlorophenoxyacetic acid |
| Dicamba | 2-methoxy-3,6-dichlorobenzoic acid |
| IAA | Indole-3-acetic acid |
| IBA | Indole-3-butyric acid |
| MCPA | 2-methyl-4-chlorophenoxyacetic acid |
| NAA | 1-naphthylacetic acid |
| NOA | 2-naphthyloxyacetic acid |
| Picloram | 4-amino-2,5,6-trichloropicolinic acid |

**1.2.2.1.1.4.Culture types**

Cultures are most commonly started from an explant a sterile piece of a whole plant. Some pieces of organs, such as leaves, roots, pollens, endosperms and embryos can be used as explants. Culture initiation is affected form many characteristics of explants. Generally, younger, more rapidly growing tissue is reliable to regeneration (Slater et al., 2008).

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When explants are cultured on an appropriate medium including auxin or available rate of auxin and cytokinin, callus formation can be exhibited. Callus is a disorganization of actively dividing and growing cells. A callus consists of a mass of loosely arranged thin-walled parenchyma cells originating from growing and developing explants. Normal roots, shoots and embryoids can be developed from callus. Callus cultures are incubated in dark to support dedifferentiation of the callus. Periodically medium refreshment is also important to maintain callus growth and dedifferentiation (Slater et al., 2008).

There are three stages of callus formation from an explants: callus induction, cell division and differentiation. It is occurred that the preparation to cell division in induction stage. During active cell division stage, calli are converted to meristematic or dedifferentiated state. At the third stage, cellular differentiation and metabolic pathways can occur (Doods and Roberts, 1985). Certain cell types can respond against *in vitro* culture conditions because of heterogeneous structure of explants.

#### 1.2.2.1.1.5. Somatic embryogenesis

Somatic embryogenesis is formed from somatic tissues. Directly or indirectly somatic embryogenesis can occur. A cell or small group of cells form directly an embryo structure without callus formation in direct somatic embryogenesis. In indirect somatic embryogenesis, firstly, callus is produced, after that, embryo is formed from produced callus tissue (Slater et al., 2008).

#### 1.2.2.1.1.6. Organogenesis

Organogenesis is the production of organs either directly from an explants or a callus culture. Natural plasticity of plant tissues and medium components using in cell culture affect the organogenesis. Generally, the auxin to cytokinin ratio of the medium determines developmental pathway of the regenerating tissue. The low level of or free auxin medium promotes shoot development. After shoot development, root can be formed simply (Slater et al., 2008).

#### 1.2.2.1.1.7.Factors affecting *in vitro* cell culture

Several factors affect *in vitro* cell culture. It is thought that the most important factor is genetic structure of explants. The culture medium needs differ from species to species and cultivars to cultivars. Media components (such as alternative carbon sources, macro- and microelement concentrations and composition), media preparation method and donor plant condition and growth conditions are other

factors affecting plant cell culture. One of the main aims of in vitro cell culture is therefore to optimize the components of medium and improve highly efficient regeneration system (Slater et al., 2008)

#### 1.2.2.1.2. Wheat tissue culture

The type of explants (Ozias-Akins and Vasil, 1982; Maddock et.al., 1983; Redway et.al, 1990; Keresa et.al, 2004), the genotype of cultivar used (Maddock et.al., 1983; Mathias, 1990; Fennel et.al, 1996; Özgen et.al, 1998; Raziuddin et.al, 2010) and tissue culture media composition (Elena and Ginzo, 1988; Fennel et.al, 1996; Yu and Wei, 2008; Miroshnichenko et.al, 2009; Ren et.al, 2010) are important factors affecting in vitro wheat tissue culture.

Genotypic differences in mature embryo based callus cultures was investigated by Sears and Decards in 1982. They reported that 2-4-D concentration can manipulate controlling of cellular organization and shoot meristems for most of genotypes. Vasil (1987) suggested that the relationship of genotype to morphogenetic competence *in vitro* is complex and indirect. "This relationship is influenced by physiological and environmental factors and has a strong effect on the synthesis, transport and the availability of plant growth regulators" (Vasil, 1987). It was also suggested that if suitable explants are excised from plants and cultured under optimal conditions with appropriate amount of plant growth regulators, plants or genotypes can be induced for morphogenesis (Vasil, 1987).

##### 1.2.2.1.2.1. Induction and maintenance of embryogenic callus

Immature embryos (Bohorava et al., 1995; Machii et al., 1998; Zhao et al., 2006; Jia et al.,2008; He et al., 2009), immature leaves (Zamora and Scott, 1983), immature inflorescences (Redway et al., 1990; Sharma et al., 1995; Demirbaş, 2004; Kavas, 2008), apical meristems (McHugen, 1983), endosperm supported embryos (Özgen et al., 1998), thin mature embryo fragments (Delporte et al., 2001) meristematic shoot

segments (Sharma et al, 2005) and mature embryos (Eapen and Rao, 1982; Ozias-Akins and Vasil, 1983; Turhan and Baser, 2003; Zale et al., 2004; Filippov et al., 2006; Bi et al., 2007; Yu and Wei, 2007; Yu and Wei, 2008; Ren et al., 2010) have been used as various explants source in wheat tissue culture.

During the initial period of excision and culture of explants in the presence of 2,4-D, embryogenic competence is expressed a few cells. Somehow, these cells are selected and preferred. The maintenance of adequate levels of 2,4-D helps to perpetuate the embryogenic nature of culture by continued divisions in embryogenic cells and in active meristematic zones formed in proliferating tissues. Lowering of 2,4-D levels results in the organization of somatic embryos. Embryogenic cells are characteristically small, thin-walled, tightly packed, richly cytoplasmic and basophilic and contain many small vacuoles as well as prominent starch grains (Vasil and Vasil, 1981). When 2,4-D levels become too low, the embryogenic cells enlarge, develop large vacuoles, lose their basophilic and richly cytoplasmic character, walls become thicker, starch disappear (Vasil and Vasil, 1982). This irreversible differentiation leads to the formation of a friable non-embryogenic callus which is generally non-morphogenic or may form roots. Most cultures are actually mixtures of embryogenic and non-embryogenic cells as a result of such continuous conversion. In general, embryogenic calli are characterized as off-white, compact, nodular type and as white compact type. Upon subculture nodular embryogenic callus was defined to become aged callus and formed an off-white, soft and friable embryogenic callus both of which retain the embryogenic capacity for many subcultures (Redway et al., 1990).

#### 1.2.2.1.2.2.Wheat regeneration system studies

A highly efficient and reproducible in vitro regeneration system is an absolute prerequisite to produce transgenic plants (Sharma et al., 2004). Immature embryos, immature inflorescences and mature embryos are the most widely used explants source in wheat regeneration system.

#### 1.2.2.1.2.2.1. Immature embryo studies

One of the earliest immature embryo regeneration was reported by Ahloowia in 1982. In this study, callus formation was good including 2,4-D and indole acetic acid and lack of kinetin. However, seed germination and fertility of plantlets were lower than embryo culture.

Callus induction and shoot formation of 39 winter wheat cultivars using one standard media series were compared by Sears and Decard in 1982. Some cultivars exhibit high regeneration rate. It was reported that the callus induction rate and regeneration was quite cultivar specific.

Ozias-Akins and Vasil (1983) produced whole wheat plant from immature embryo and immature inflorescences in including 2 mg/l 2,4-D medium. They also reported that plants were regenerated only from the compact callus and their chromosome number was normal.

The effect of the interaction of genotype and culture medium on the initiation of callus from immature embryos and plant regeneration in 8 hexaploid wheat lines was reported by Mathias and Simpson in 1987. Coconut milk was used as an organic additive. It increased shoot and primordia development for some cultivars and inhibited for others. They suggested that the response of culture depends on genotype.

Borrelli and his group (1991) compared the response of callus derived from scutellum of immature embryos of five durum varieties growing in semi-arid mediterranean areas presence of 2,4-D. They used hormone free medium for plant regeneration. Italian group suggested that three of them have the best response and they could be used for biotechnological approaches.

Arzani and Miradjogh (1999) evaluated 28 durum wheat cultivars immature embryo derived callus production and in vitro salt tolerance. They reported that two wheat genotype were more tolerant than others.

Przetakiewicz and colleagues (2003) compared of the role of three different auxins on somatic embryogenesis and plant regeneration in three different cereal species. Eight cultivars of barley, five cultivars of wheat and three cultivars of triticale were evaluated. Two different media were used in this study to determine plant regeneration. They reported that the type of plant growth regulators used in induction medium and the type of regeneration medium affect the regenerated plantlets number.

Pellegrineschi and co-workers (2004) obtained optimal callus induction and plant regeneration from bread and durum wheat by manipulating the concentration of NaCl in induction medium. They found high callus induction and plant regeneration rate from including 2 mg/l 2,4-D induction medium for bread wheat. For durum wheat, immature embryos were incubated in 2 mg/l 2,4-D and 2 mg/l NaCl induction medium.

Hungarian winter wheat immature embryos were used to determine the effect of growth regulators, macroelements used in regeneration medium, the incubation temperature and the light density and their combination on regeneration frequency by Tamas and colleagues in 2004.

Haliloglu and Baenziger (2005) evaluated the response of immature embryos of three spring and five winter cultivars for three callus induction media. Media were varied according to including plant growth regulators (2,4-D and Picloram), vitamins and their combination or not. The medium containing 2.2 mg/ml picloram and 0.5 mg/ml 2,4-D and MS vitamins called CM4C gave the best results for embryogenic callus formation.

Wu and co-workers (2006) found that silver nitrate ($AgNO_3$) prevents necrosis and increases callus growth of derived from immature embryos of four wheat cultivars. They suggested that 10 mg/l silver nitrate may have good effect to prevent necrosis and promote callus growth.

Chauhan and colleagues (2007) developed genotype independent, in vitro cell culture regeneration system for nine Indian wheat cultivars manipulating the concentration and exposure time to the growth regulators and thidiazuron. They achieved 80 % of regeneration rate using these combinations. It was suggested that the low concentration of thidiazuron and auxin combination have the best effect of regeneration of immature and mature embryo based callus.

Dağüstü (2008) investigated the capacity of callus formation and plant regeneration from immature embryo cultures of seventeen winter wheat genotypes. Compact and frequently embryogenic or watery and soft callus were evaluated. It was emphasized that the regeneration rate of compact callus was higher than soft callus.

Miroshnichenko and colleagues (2009) found that addition to medium the low concentration of diaminozide has positive effect the somatic embryogenesis capacity of immature embryo. They reported that the higher level of diaminozide has reductive effect for shoot formation.

Koescielnick and co-workers (2010) tested zearalenone and thidiazuron activity combination with 2,4-D using wheat immature embryo cultures. They proposed that zearalenone could be used as a new growth regulator in in vitro cell culture studies.

#### 1.2.2.1.2.2.2. Immature inflorescences

Ozias-Akins and Vasil (1982) reported that the compact, yellowish and nodular callus formed from the rachis and glumes of immature inflorescences using 2 mg/l 2,4-D with MS medium.

Eapen and Rao (1984) used immature inflorescences from wheat, rye and triticale to initiate callus formation. Marcinska and colleagues (1995) investigated two different wheat varieties callus formation capability.

Barro and co-workers (1999) developed media to use in somatic embryogenesis and plant regeneration from immature inflorescences and immature scutellum of elite cultivars of wheat, barley and tritordeum. They reported that had two fold effect than 2,4-D for immature inflorescences based callus formation. They also reported that the addition of zeatin to regeneration medium had positive effect on plant regeneration

Caswell and co-workers (2000) used for Canadian wheat cultivars immature inflorescences to regenerate healthy plants. They tested three sizes of immature inflorescences with two different media. Benkirane and colleagues (2000) investigated somatic embryogenesis and plant regeneration of ten durum wheat. They used two different concentration of 2,4-D. they achieved to produce embryogenic callus and subsequently fertile plants.

Durusu (2001) compared eight Turkish wheat cultivars according to their embryogenic capacities using immature embryos and immature inflorescences derived callus. It was reported that immature inflorescenceshad more callus formation potential than immature embryos.

Keresa and colleagues (2004) compared callus induction and plant regeneration response from eight Crotian wheat cultivars. Immature embryos, immature inflorescences and mature embryos used as explants source. They reported that including picloram media gave the best results for immature inflorescences.

Demirbaş (2004) optimized regeneration of Turkish wheat cultivar, Yüreğir 89. The different parts of immature inflorescences were used as explants source. The

embryogenic capacity and regeneration of different parts of immature inflorescences were determined.

Kavas (2008) studied on optimization of tissue culture and regeneration of two winter cultivars. It was detected that vernalization period of donor plants were important factor affecting in vitro cell culture of winter wheat cultivars. Two different media were used for callus induction. It was reported that 100 % callus induction rate was observed for two cultivars.

#### 1.2.2.1.2.2.3. Mature embryos

O'Hara and Street (1978) are researchers studying firstly mature embryos from nodal and inter nodal segments of stem and from rachis segment incubated on MS medium with 1 mg/l 2,4-D growth regulator. They claimed that callus yield was related with used cultivars. They also suggested that presence of auxins in medium was essential for callus formation

Eapen and Rao (1982) investigated different type of auxins on formation of callus from mature embryos of durum and emmer wheat. Coconut milk and NAA were used as additive to basal medium. They produced whole plant and harvested seeds.

Ozias-Akins and Vasil (1983) studied callus induction and growth from the mature embryo of wheat. They tested different concentration of 2,4-D. they claimed that if the concentration of 2,4-D was equal or greater than 2 mg/l , cell differentiation could be initiated. However, higher concentration of 2,4-D had an inhibitive effect to cell proliferation.

Özgen and colleagues (1998) developed an efficient method for callus induction and plant regeneration from mature embryos of twelve common winter wheats. They used endosperm supported embryo culture technique. Including 8 mg/l 2,4-D medium was used for callus initiation. For regeneration, developed calli were

incubated on hormone free medium. They claimed that mature embryo had a high frequency of callus induction and regeneration capacity.

Varshney and colleagues (1999) used mature embryos of seventeen cultivar of bread wheat and three cultivar of durum wheat. They compared their embryogenic callus formation capacity. Different concentration of 2,4-D and some organic additives were used to increase callus response. They produced healthy plants and harvested seeds.

Gopalakrishan and colleagues (2001) compared twenty different growth regulator combinations using mature embryos of two wheat genotypes. They did not observed callus formation in hormone free or low concentration of plant growth regulators. Optimum callus growth was observed including 2 mg/l 2,4-D medium. They supported regeneration medium with BAP and IAA. Hormone free medium was used for root formation. They produced fertile and viable seeds.

Özgen and colleagues (2001) studied on cytoplasmic effects of embryo culture responses using calli derived from mature embryos from four bread wheat cultivars. They found that cytoplasm positively affected callus formation, regeneration capacity of callus, culture efficiency and numbers of regenerated plants. They investigated that the effect of cytoplasm was related with genotype.

Delporte and colleagues (2001) developed a wheat regeneration system using thin mature embryo fragments of wheat. The callus induction medium was supplemented with 2,4-D. They produced 513 plantlets using this method.

Mendoza and Kaeppler (2002) compared auxin and sugar effects on callus induction and plant regeneration frequencies from mature embryos of wheat. They used four different auxin (2,4-D, picloram, dicamba and propionic acid) and two sugar (sucrose and maltose).

Turhan and Baser (2003) evaluated two different methods for callus formation from mature embryos of winter wheat. They supported five media with different concentration of 2,4-D and NAA.

Kilinc (2004) evaluated effect of dicamba on the embryo cultures of seven bread wheat. It was claimed that the response of culture depended on th genotypes and dicamba concentrations. It was observed that the highest callus formation rate was 63.1 % in including 5 mg/l dicamba with Linsmaier Skoog medium

Filippov and colleagues (2006) evaluated the effect of auxins, exposure time to auxin and genotypes on somatic embryogenesis and plant regeneration of Russian wheat cultivars mature embryo. They achieved the highest value of embryogenic callus formation and plant regeneration rate including 12 mg/l dicamba medium.

Bi and colleagues (2007) compared callus formation and subsequently regeneration of embryo cultures of thirty-one plants of different *Triticum* species. They found significant differences in callus induction, embryogenic callus formation, plant regeneration and culture efficiency. They produced plantlets from these genotypes.
Yu and colleagues (2007) developed a new method for embryo based tissue culture of wheat. They found that the MS medium combination and longitudinally bisected embryos gave the highest culture efficiency. They achieved 70 % of primary callus induction in all tested cultivars using 2 mg/l 2,4-D in basal medium. They observed that the culture efficiency varied from 15.3 % to 36.8%.

Yu and Wei (2008) evaluated effects of cefotaxime and carbenicillin on plant regeneration from wheat mature embryos. They reported that filter-sterilized cefotaxime increased regeneration capacity. However, it decreased the average shoot number of per explants. They also reported that carbenicillin did not affect plant regeneration.

Chen and colleagues (2009) analyzed expression of auxin related genes from wheat callus derived mature embryo using affimetrix microarray technique. The embryos were incubated on including 2 mg/l 2,4-D medium. 2, 4, 12, 24 and 72 hours incubated explants were evaluated. They found that 80 auxin related genes, 41 of them up-regulated, 29 of them down-regulated and 10 of them both up- and down-regulated genes. They reported that these genes were related with several biological processes, such as the transportation, response, induction, synthesis and degeneration of auxin.

Although the most frequently used explants sources are immature embryos for plant regeneration from callus culture of wheat and have the highest rates of callus induction and plant regeneration, the use of immature embryos is limited. Because they cannot be supplied throughout the year and their most suitable stage for efficient culture is also strictly limited, inhibiting their application for *in vitro* culture and genetic transformation. A regeneration system based on mature embryos may overcome these limitations, as they can be stored in the form of dried seeds and are readily available all the time. Furthermore, the physiological state of mature embryos shows minimal variability, an important trait for plant tissue culture (Yu et al., 2008).

#### 1.2.2.2.Wheat transformation

Wheat improvement with desired traits by genetic engineering requires the delivery, integration and expression of defined foreign genes into suitable regerenable explants (Patnaik and Khurana, 2001). The first studies about fertile transgenic plants belong to last two decades (Vasil et al, 1992, 1993; Weeks et al., 1993; Becker et al., 1994; Nehra et al., 1994). Genotype, growth conditions of donor plants, tissue culture and transformation method affect producing fertile transgenic plants.

After delivery of a gene cassette into recipient cells, the expression of delivered gene is analyzed. The expression can be detected using e reporter gene into delivered gene cassette (Patnaik and Khurana, 2001). The reporter genes produce a visible effect,

directly or indirectly, due to their activity in the transformed cells. β-glucuronidase (*gus*) gene from *E.coli* is the most widely used in wheat transformation (Vasil et al., 1992; Vasil et al.,1993; Weeks et al., 1993; Becker et al., 1994). β-glucuronide compounds are hydrolyzed by GUS enzyme. This reaction can be detected spectrophotometric or spectrofluorometrically (Jefferson et al., 1987). The *gus* reporter gene system is extremely useful to optimize genetic transformation parameters. Because, the reaction results can be observed aid of a simple histochemical assay. *cat* gene from *E.coli* encoding chloramphenicol acetyl transferase (Hauptmann et al., 1988; Chibbar et al., 1991), green fluorescent protein (*gfp*) from jellyfish (Pang et al., 1996, McCormac et al., 1998), R genes from *Zea mays* (Kloti et al.,1993; Chawla et al., 1998) and luciferase gene from *Photinus pyranus* (Lonsdale et al., 1998; Harvey et al., 1999)are other scorable markers used in wheat transformation successfully (Patnaik and Khurana, 2001).

The varied frequency of DNA delivery in cells of different explants has necessitated the development of methods for efficient selection of cells that carry and express the introduced gene sequences. The selection regimes for transformed cells are based on the expression of a gene termed as the selectable marker producing an enzyme that confers resistance to a cytotoxic substance often an antibiotic or a herbicide. The most commonly used selection marker in wheat transformation is the *bar* (Murakami et al., 1986; Thompson et al., 1987) (bialaphos resistance gene) and *pat* (Wholleben et al., 1988) genes encoding for phosphinothricin acetyl transferase (PAT). Both *bar* and *pat* genes isolated from different *Streptomyces* species, encode for phosphinothricin acetyl transferase. Amongst the antibiotic resistance markers, the bacterial neomycin phosphotransferase II (*npt*II) gene providing resistance to aminoglycoside antibiotics is commonly used in wheat transformation. Herbicide resistance genes offer an alternative to antibiotic-resistant markers. Aminoglycoside based antibiotics is commonly used in wheat transformation. Herbicide resistance genes offer an alternative to antibiotic-resistant markers (Patnaik and Khurana, 2001). 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), enolpyruvylshikimate-phosphate synthase (*CP4*) gene (Zhou et al., 1995),

hygromycin phosphotransferase (*hpt)* gene (Ortiz et al., 1996), mannose-6-phosphate isomerase (MPI) (Hansen and Wright, 1999) and cyanamide hydratase (*Cah*) gene (Weeks, 2000) are the other selectable marker used in wheat transformation.

Phosphinotricin (PPT) and aminoglycoside based antibiotics are the most widely used selection agents in wheat transformation (Goodwin et al., 2004). Glutamine synthetase has a very important role for ammonium assimilation and nitrogen metabolism in plants (Deblock et al., 1987). PPT inhibits irreversibly this enzyme synthesis and causes increase of ammonia level for toxic to cells (Tachibana et al., 1986). Phosphinotricin acetyl transferase converts PPT to the non-toxic acetylated form and allows the growth of transformed cells in the presence of PPT or commercially available glufosinate ammonium. Kanamycin, neomycin, gentamycin, G418 and hygromycin are used as selection agent (Jones, 2005).

Immature inflorescences (Demirbas, 2004; Kavas, 2005), immature embryos (Vasil et al., 1993; Varshney and Altpeter, 2002) and mature embryos (Öktem et al., 1999; Patnaik and Khurana, 2003) are the most widely used explants in wheat genetic transformation.

Electroporation, micro-injection, silicon carbide fibres, polyethylene glycol, laser mediated uptake, floral dip transformation, particle bombardment or biolistic and *Agrobacterium* mediated are the techniques used in wheat transformation varying degrees of success. However, particle bombardment and *Agrobacterium* mediated are the most widely used two techniques.

#### 1.2.2.2.1. *Agrobacterium*- mediated wheat transformation

*Agrobacterium tumefaciens* is a soil pathogen bacterium and causes crown gall tumor disease in dicotyledonous plants. It can transfer a small fragment of its DNA (T-DNA) of tumor inducing (Ti) plasmid to its host plant cell (Nester et al., 1984; Binns and Thomashaw, 1988). There are two types of genes on T-DNA; the oncogenic

genes, and opines synthesis genes. The oncogenic genes encodes for enzymes involved in the synthesis of auxins and cytokinins and responsible for tumor formation. Opines, produced by condensation between amino acids and sugars, are synthesized and discharged by the crown gall cells and consumed by *A. tumefaciens* as carbon and nitrogen sources. Outside the T-DNA, are located the genes for the opine catabolism, the genes involved in the process of T-DNA transfer from the bacterium to the plant cell and the genes involved in bacterium-bacterium plasmid conjugative transfer. (Hooykaas and Schilperoort, 1992; Zupan and Zambrysky, 1995). *Agrobacterium*- mediated transformation is a simple, low cost and highly efficient alternative to direct gene delivery methods (Patnaik and Khurana, 2001).

Hess and colleagues (1990) reported the first *Agrobacterium* transformation to wheat. They achieved to transfer kanamycin resistance via pipetting bacteria onto spikelets.

Amoah and colleagues (2001) studied on factors effecting *Agrobacterium*- mediated transient expression of *uidA* gene in wheat inflorescences tissue. They used AGL1 strain of *Agrobacterium*. They optimized duration of preculture, vacuum infiltration, the effect of sonication treatments and *Agrobacterium* cell density.

Wang and colleagues (2002) tested immature embryos and mature embryos callus from two winter wheat cultivars with three strain of *Agrobacterium tumefaciens* AGL1, EHA 105 and LBA4404. They used *bar* gene as a selectable marker and *gus* gene as a scorable marker. They reported higher osmotic treatments affected positively transformation rate. They claimed the strain of bacterium, receptor genotype, explants type and its age and physiological state affected transgenic plant regeneration. The production of transgenic plants was corrected using PCR and Southern blot analysis.

In 2006, Patnaik and colleagues developed a highly efficient and reproducible method using *Agrobacterium* transformation. Mature embryos of bread and winter

wheat were used explants. They achieved to increase transient expression of *gus* gene 1.5-2.0 fold with presence of acetosyringone in the bacterial growth medium, co-cultivation medium and inoculation medium. They produced $T_0$ and $T_1$ plants and did not observe any differences on PCR amplification and Southern hybridization.

Himmelbach and colleagues (2007) offered the vector set with a series of functionally validated promoters and allows for rapid integration of the desired genes or gene fragments by GATEWAY based recombination. They used a versatile set of binary vectors for transgene overexpression, as well as for gene silencing by double stranded RNA interference.

Wu and colleagues (2009) investigated efficient and rapid *Agrobacterium*-mediated transformation of durum wheat using additional virulence genes. They used AGL1 strain of *Agrobacterium* with vir genes introduced a helper plasmid. It was observed the transformation frequency between 0.6 and 9.7 %. They claimed this study was the first successful genetic transformation of tetraploid durum wheat using *Agrobacterium*-mediated gene delivery method.

In 2009, Wang and colleagues used mature embryos of spring and winter wheat to observe transient g*us* expression using *Agrobacterium*-mediated transformation. They exhibited the presence of the antibiotic selection marker in the $T_0$ plants via genomic PCR amplification and enzyme-linked immunosorbent assay (ELISA).

He and colleagues (2010) developed efficient *Agrobacterium*-mediated durum wheat transformation system. They used *Agrobacterium* strain AGL1 containing super binary vector system and durum wheat immature embryos. They evaluated effects of acetosyringone and picloram. They reported the higher concentration of acetosyringone and picloram (10 mg/l) increased transformation frequency. Southern blot analysis, GUS assay and genetic analysis were used to confirm stable integration of foreign genes, their expression and inheritance.

#### 1.2.2.2.2. Particle bombardment transformation studies in wheat

Particle or microprojectile bombardment (also called biolistic) involves the adsorption of plasmid or linear forms of naked DNA onto surface of submicron particles of gold or tungsten which are driven at high velocity into recipient plant cells using an acceleration device (Sanford, 1988; Sanford et al., 1993; Jones, 2005). The development of methodologies for the delivery of genes into intact plant tissues by particle bombardment has, in fact, revolutionized the field of plant transformation. This method of introducing DNA into cells by physical means was developed to overcome the biological limitations of *Agrobacterium* and difficulties associated with plant regeneration from protoplasts (Patnaik and Khurana, 2001). Biolistic has also been used to deliver DNA into the chloroplast and mitochondrion genomes and effective DNA- transfer has also been demonstrated using *Escherichia coli* and *Agrobacterium* cells as microprojectiles (Rasmussen, 1994).

Particle bombardment effectively distributes DNA over a wide area of the target tissue and is relatively genotype independent. However several parameters must be optimized for particular explants including the microprojectile type, size, and quantity; DNA quantity and method of precipitation; and the acceleration device parameters such as propellant force, helium pressure and target distance. All of these parameters can influence the efficiency of DNA delivery and the extent of damage to the explants tissues (Altpeter et al., 1996; Harwood et al, 2000; Ingram et. A,1999; Perl et al., 1992; Rasco-Gaunt et al., 1999).

Particle bombardment method is successfully being used for the generation of transformed wheat with introduction of agronomically important genes for quality improvement, engineering of nuclear male sterility, transposon tagging, resistance to drought stress, resistance to fungal diseases and insect resistance (Patnaik and Khurana, 2001). Agronomically important genes introduced into wheat via particle bombardment are demonstrated in Appendix B.

Although recent advances in *Agrobacterium*- mediated transformation of plants, DNA transfer via particle bombardment is still a widely used method for nuclear transformation of many monocot and dicot species (Sivamani et al., 2009).

Wang and colleagues (1988) investigated transient gene expression of foreign genes in rice, wheat and soy bean cells following particle bombardment. They directly bombarded including tungsten particles coated with plasmids including β-glucuronidase gene into the intact cells. They defined optimum conditions detecting blue transformed cells via enzyme assay.

In 1991, Vasil and colleagues obtained stably transformed callus lines by direct delivery of DNA into plated suspension culture cells of wheat using high velocity of microprojectile bombardment. They used three different reporter or selectable genes. One-year later, 1992, Vasil and colleagues obtained the first fertile transgenic wheat plants resistant to herbicide basta by particle bombardment.

In 1994, Takumi and colleagues studied on effect of six promoter-intron combinations on transient reporter gene expression in einkorn, emmer and common wheat cells by particle bombardment. They used four promoters; cauliflower mosaic virus (CaMV) 35S, tandem (CaMV35S), maize alcohol dehydrogenase gene (*Adh1*) and rice actin gene (*Act1*) promoters and two introns *Adh1* intron 1 and castor bean catalase intron. The lowest level of transient expression in wheat cells was CaMV35S promoter. They suggested the rice *Act1* promoter giving the highest transient expression level was efficient for use in wheat transformation.

Altpeter and colleagues (1996) developed a method for the accelerated production of fertile transgenic wheat plants ready for transfer to soil in 8-9 weeks after the initiation of cultures. They manipulated culture, bombardment and selection procedures. 4-6 hours pre- and 16 hours post- bombardment osmotic treatment were applied to cultured immature embryos. They reported the highest rates of regeneration and transformation were obtained when callus formation after

bombardment was limited to two weeks in dark, with or without selection, followed by selection during regeneration under light. They produced eight independent transgenic wheat lines.

In 1997, Takumi and Shamada reported the first transgenic durum wheat using particle bombardment of scutellar tissues. They used plasmid pDM302 containing the biaphalose resistant gene (*bar*) under control of the rice actin 1 gene (*Act1*) promoter. The confirmation of transgenic plants was performed PCR amplification and Southern blot analysis in $T_0$ and $T_1$ plants. The frequency of transformation was 1.17%.

Chen and colleagues (1997) bombarded the plasmid pBI121 via particle bombardment into immature embryo of four spring wheat cultivars. They studied on transformation parameters such as the bombardment velocity, tungsten particle diameter, DNA- coated particle loading way and DNA concentration. They confirmed the presence of β-glucuronidase gene in transgenic lines via Southern blot analysis.

Liang and colleagues (1998) investigated some factors affecting transformation of wheat immature embryos by particle bombardment. They cultured two weeks old calli on mannitol added medium 6 hours pre- and 18 hours post- bombardment. They reported this incubation increased transient gus expression several fold.

In 1999, Rasco-Gaunt and colleagues analyzed particle bombardment parameters to optimize DNA delivery into wheat tissues. They tested the DNA/gold precipitation process, bombardment parameters and tissue culture variabilities. They also analyzed amount of DNA, spermidine concentration, presence of calcium ions, calcium chloride concentration, amount of gold particles, gold particle size, acceleration pressur, chamber vacuum pressure, bombardment distance, osmotic conditioning of tissues and type of auxin.

Öktem and colleagues (1999) investigated marker gene delivery to mature embryo via particle bombardment. The plasmid (pBSGUSINT) was coated tungsten particles. They tested various gas pressure and in a chamber vacuum. They observed 80 % of transient gene expression frequency from bombarded embryos.

In 2001, Wright and colleagues developed a new selection system using phosphomannose isomerase (*pmi)* gene in biolistic transformation. They reported this system did not require the use of antibiotic and herbicide resistance genes. The selectable marker consisted of the *manA* gene from *E.coli* under the control of a plant promoter. They reported transgenic plants metabolized the selection agent, mannose, into a usable source of carbon, fructose. They claimed the frequency of transformation was 20 % for wheat, 45 % for maize.

In 2003, Patnaik and Khurana investigated genetic transformation of Indian bread and pasta wheat by particle bombardment of mature embryo derived calli. Calli were double bombarded with 1. gold1 microprojectiles coated with pDM302 and pAct1-F at a target distance of 6 cm. $T_0$ transformants were confirmed by Southern blot analysis. The *bar* gene activity was observed in $T_0$ and $T_1$ plants via phosphinotricin leaf point assay. They reported the frequency of transformation was 8.56 % for bread wheat and 10 % for durum wheat.

Delporte and colleagues (2005) investigated microprojectile DNA delivery into callus initiated from mature embryo fragments. The *bar* and *gus* genes were used as selectable and reporter marker genes. They reported 6 day cultivation before bombardment was optimum condition for DNA uptake and β-glucuronidase expression.

Yao and colleagues (2007) optimized wheat co- transformation procedure with gene cassettes resulted in an improvement in transformation frequency. They simultaneously transferred to wheat immature embryos with two non- linked genes, *gus*and *bar*, or one plasmid. They observed no differences in GUS transient

expression of between gene cassettes and single plasmid. However, the stable gene transformation frequency was significantly increase to 1.1 % using gene cassettes.

Xing and colleagues (2008) studied on transformation of wheat thaumatin-like protein (Ta-Tlp) gene and analyzed reactions to powdery mildew and *Fusarium* head blight in transgenic wheat plants. The vector pAHC-Tlp constracted was transformed into immature embryo derived calli through particle bombardment. They confirmed the *Ta-Tıp* gene integration into the wheat genome and expression in $T_1$ and $T_2$ generation using PCR, Southern blot and RT-PCR analysis. They also performed biologic assay inoculating $T_0$, $T_1$ and $T_2$ with *Erysiphe aminis* and *Fusarium graminearum* for resistance identification. All plants of $T_0$, $T_1$ and $T_2$ generations were resistant to wheat powdery mildew by delaying disease development, but no distinct resistance to *Fusarium* head blight.

Tamas and colleagues (2009) investigated amaranth albumin gene, encoding 35-kDa AmA1 protein of the seed, with a high content of essential amino acids to improve wheat nutritional quality. They bombarded this gene to bread wheat via particle bombardment. They confirmed the integration of gene with Southern blot analysis. They reported the results exhibited that not only can essential amino acids content be increased, but some parameters associated with functional quality may also be improved because of the expression of the AmA1 protein.

### 1.3. Aim of the study

The main objective of this study is the optimization of mature embryo based regeneration systems for two Turkish wheat cultivars (Mirzabey 2000 and Yüreğir 89). For these reasons following approaches have been considered:

i. determination of effect of hormone types, concentrations and dark incubation period,

ii. determination of correlation of hormone type, concentration, dark incubation and genotype on primary callus induction, embryogenic callus formation, regeneration, culture efficiency, shoot formation and root formation,
iii. optimization of particle bombardment with pAHC25 coated particles to mature embryo derived callus.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1.Materials

### 2.1.1. Plant materials

In this study, mature embryos of winter durum wheat *Triticum durum* cultivar Mirzabey 2000 and spring bread wheat *Triticum aestivum* cultivar Yüreğir 89 were used. The seeds of Mirzabey 2000 were obtained from Agricultural Research Institute, Ankara. The seeds of Yüreğir 89 were kindly provided by Çukurova Agricultural Research Institute, Adana. More information about cultivars was given in Appendix A.

### 2.1.2. Chemicals

Chemicals used in this study were provided by Sigma-Aldrich Company (New York, USA), Applichem (Darmstadt, Germany), Duchefa (Haarlem, Holland), PhytoTechnology Laboratories (USA), Fermentas (Burlington, Canada). Distilled water was used to prepare solutions.

### 2.1.3. Plant tissue culture media

In tissue culture studies, four different media were used: i. Callus Induction Medium, ii. Embryogenic Callus Formation Medium, iii. Regeneration Medium and iv. Root Strength Medium. All media were composed of MS Basal Salts with vitamins

(Murashige and Skoog, 1962), sucrose and phytagel. The Table 2.1 shows composition of media.

#### 2.1.3.1. Callus induction medium

Callus induction medium includes 4.4 g/l MS Basal Salts With Organics (PhytoTechnology Laboratories, USA), 30 g/l sucrose, 134 mg/l L-aspartic acid, 146 mg/l L-glutamine, 115 mg/l L-proline, 100 mg/l casein hydrolysate and 40 mg/l L-tryptophane (Yu et.al, 2008). MS Basal Salts with vitamins, sucrose and other additive organics were dissolved in distilled water. pH of the solution was adjusted to 5.8 with 1 M NaOH solution. After that 0.3 % phytagel was added to solution for medium solidification. Medium was autoclaved at 121˚C for 20 minutes, and medium warm, picloram and 2,4-dichlorophenoxyacetic acid (2,4-D) were added to medium, then poured into sterile plastic petri dishes under sterile conditions.

#### 2.1.3.2. Embryogenic callus formation medium

Embryogenic callus formation medium is same with callus induction medium. However, 1-Naphthaleneacetic acid (NAA) and 6-Benzylaminopurine (BAP) were added to medium together with picloram and 2, 4-D.

#### 2.1.3.3. Regeneration medium

Regeneration medium is same with callus induction medium. After sterilization, plant hormones were not added to medium. To increase regeneration, silver nitrate ($AgNO_3$) and cupper sulphate ($CuSO_4$) were added to medium.

#### 2.1.3.4. Root strength medium

Root strength medium includes 2.2 g/l MS Basal Salts with vitamins, 20 g/l sucrose and 0,3% phytagel. Root strength medium was poured in sterilized 1 liter glass jars.

Table 2. 1. Tissue culture media composition

| **Chemicals** | **Callus induction medium** | **Embryogenic callus induction medium** | **Regeneration medium** | **Root strength medium** |
|---|---|---|---|---|
| MS (g/l) | 4.4 | 4.4 | 4.4 | 2.2 |
| L-Aspartic acid (mg/l) | 135 | 135 | 135 | - |
| L-Glutamine (mg/l) | 150 | 150 | 150 | - |
| L-Proline (mg/l) | 115 | 115 | 115 | - |
| Casein hydrolysate(mg/l) | 100 | 100 | 100 | - |
| L-Tryptophane (mg/l) | 40 | 40 | 40 | - |
| Sucrose (g/l) | 30 | 30 | 30 | 20 |
| Phytagel (g/l) | 2.6 | 2.6 | 2.6 | 2.6 |
| Auxin (2,4-D or picloram) (mg/l) | 2 or 4 or 8 | 2 or 4 or 8 | - | - |
| Naphtalaacetic acid (mg/l) | - | 0.1 | - | - |
| Benzyl adenine (mg/l) | - | 0.5 | - | - |
| $AgNO_3$ (mg/l) | - | - | 10 | - |
| $CuSO_4$ (mg/l) | - | - | 2 | - |

### 2.1.4. Transformation

Obitek Biolab Gene Transfer System (Figure 2.1) produced in Turkey was used in this study. DNA was coated on 1µm gold particles. 14 days old mature embryo derived calli were used plant explants. GUS solution was used for determination of transient expression. The chemical components of GUS solution was given in Appendix D.

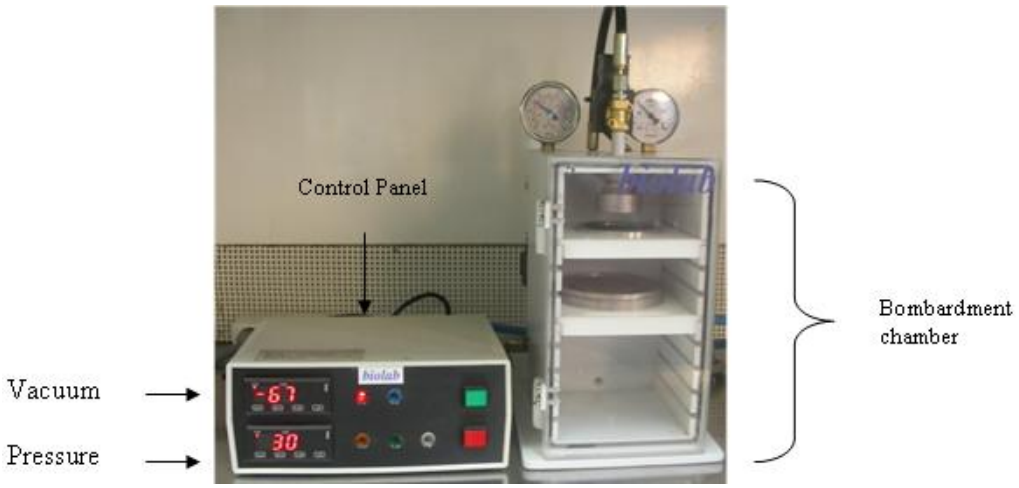


Figure 2. 1.Obitek Biolab Gene Transfer System

#### 2.1.4.1. Bacterial strain and plasmid

In genetic transformation studies, plasmid pAHC25 (Figure 2.2) was used. It includes GUS gene as a visual marker and *bar* gene as a plant selectable marker. *Echerichia coli* DH5α strain was used as a bacterial strain. pAHC25 was kindly provided by Dr. Tepperman.

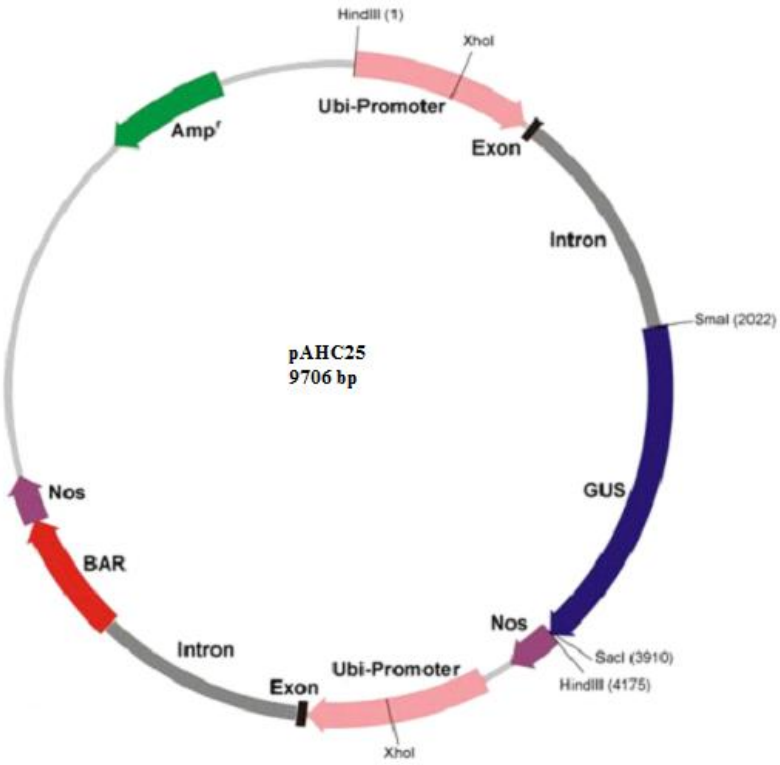


Figure 2. 2.The map of plasmid

### 2.1.4.2. Bacterial medium

Luria Broth medium (L3022, Sigma) was used to grow bacteria. Bacterial medium components were given in Appendix E.

## 2.2.Methods

### 2.2.1. Tissue culture studies in wheat

The effects of two different plant growth hormones in three different concentrations and two different dark incubation periods were investigated for two Turkish wheat cultivar regeneration capacities. 12 different media were tested and they were called

according to different treatment (Table 2.2). Schematic design of experiment was shown in Figure 2.4.

#### 2.2.1.1. Seed surface sterilization and imbibition

Mature wheat seeds were surface-sterilized with 70 % (vol/vol) ethanol for five minutes and then rinsed with sterile distilled water for five times. After that, seeds were treated with 30 % (vol/vol) NaOCl for twenty minutes, followed by five times sterile distilled water. They were imbibed with 8 mg/l 2,4-D solution at 4˚C for 16-20 hours. After imbibition, seeds were again sterilized with 70 % (vol/vol) ethanol for one minute and five times washed with sterile distilled water (Bi et al, 2007).

#### 2.2.1.2. Isolation of mature embryos from seeds

Mature embryos were aseptically removed from imbibed seeds using blade and forceps under stereomicroscope (Figure 2.3). The instruments were sterilized at 250˚C in sterilizer. Radical portion of mature embryo was slightly damaged and cultured with scutellum in contact with the medium to start initiation of callus formation. 15 mature embryo explants were cultured on each petri dishs.

#### 2.2.1.3. Callus induction and maintenance

Two different plant growth hormones, picloram and 2,4-D, were used with three different concentrations 2 mg/l, 4 mg/l, and 8 mg/l to optimize the best regeneration condition. Mature embryos in contact with medium were incubated for 4 and 6 weeks dark incubation at 25 ˚C. Media were refreshed every two weeks. After dark incubation periods, calli were weighted and carried out to the embryogenic callus formation medium. Callus induction rate was also determined as follow;

Callus induction % = Number of callus / Number of cultured mature embryo X 100

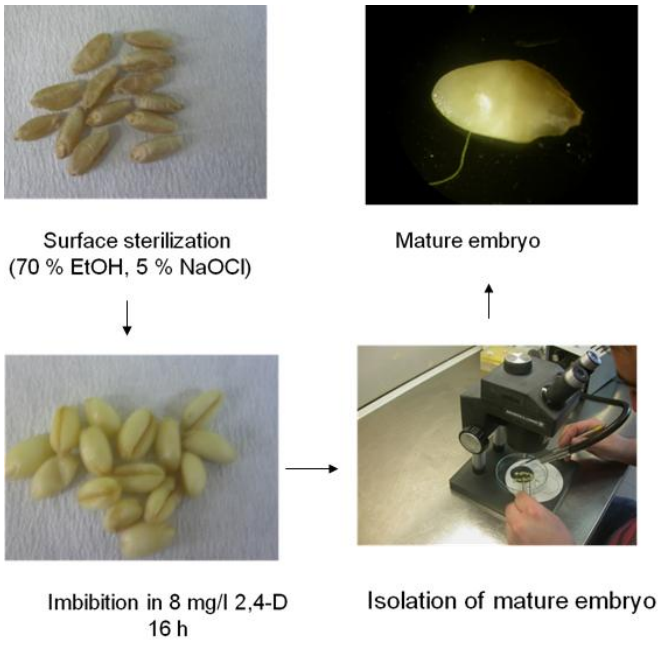


Figure 2. 3. Isolation of mature embryo

### 2.2.1.4. Embryogenic callus formation

In addition to plant growth hormones, 0.1 mg/l NAA and 0.5 mg/l BAP were added to the medium to increase embryogenic callus formation (Yu, et. al., 2008). Media were refreshed bi-weekly periods and incubated at 25 ˚C under light condition 2000 lux. After 4 weeks embryogenic callus formation medium incubation, calli were weighted. Embryogenic callus rate was calculated as follow;

Embryogenic callus induction % = Number of Embryogenic callus / Number of Callus transferred from callus induction medium X 100

### 2.2.1.5. Plant regeneration and root strength

Embryogenic callus were incubated at 25 ˚C and 3000 lux light condition. Regeneration rate, culture efficiency, shoot formation rate and root formation rate were calculated as follow;

Regeneration Rate % = Number of regenerated callus / Number of embryogenic callus X 100

Culture efficiency % = Number of regenerated callus / Number of cultured mature embryo X 100

Shoot Formation Rate % = Number of shoot / Number of regenerated mature embryo X 100

Root Formation rate % = Number of callus with root / Number of cultured mature embryo X 100

After shoot development, calli were transferred to root strength medium**.** 4 weeks later, plantlets were planted to 1:1 soil and torf mixture previously autoclaved and cooled to prevent any contamination and weed formation. For acclimatization, pots containing plantlets were covered with plastic bags to maintain humid environment conditions. After 5 days, holes were punched on the plastic bags to help the plants to become accustomed to the greenhouse conditions gradually. Ten days after the transfer, plastic bags completely removed from pots. Mirzabey cultivar needs to vernalization period. Mirzabey cultivar was transferred to vernalization room which temperature +4°C for 4 weeks. After 4 weeks vernelization period, plants were again transferred to greenhouse. Plants were watered regularly.

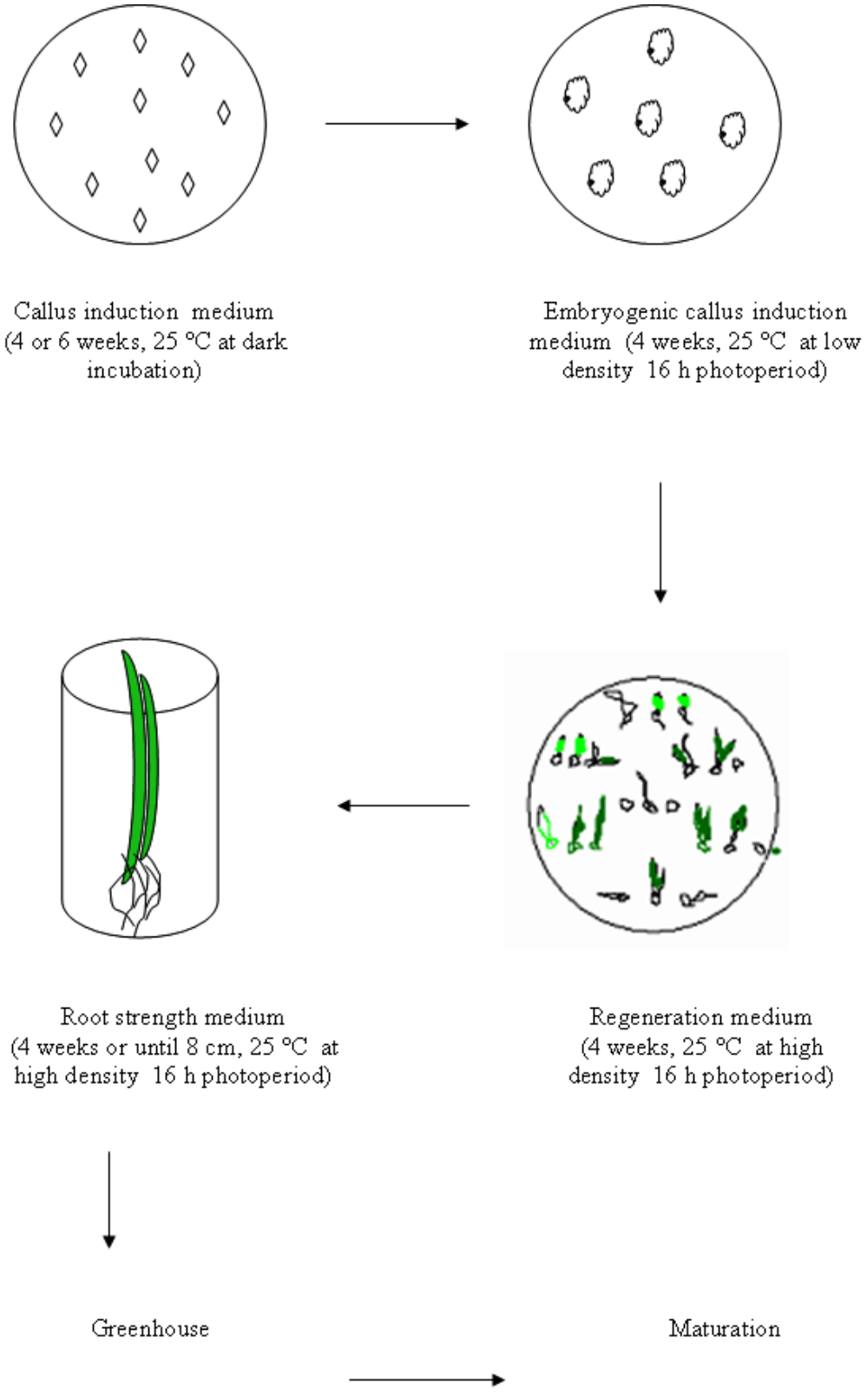


Figure 2. 4. Schematic presentations of tissue culture experiment

Table 2. 2. Media and the parameters in tissue culture

| **Medium** | **Dark Incubation (Week)** | **Auxin Type** | **Concentration (mg/l)** |
|---|---|---|---|
| 4W2D | 4 | 2,4-D | 2 |
| 4W4D | 4 | 2,4-D | 4 |
| 4W8D | 4 | 2,4-D | 8 |
| 4W2P | 4 | Picloram | 2 |
| 4W4P | 4 | Picloram | 4 |
| 4W8P | 4 | Picloram | 8 |
| 6W2D | 6 | 2,4-D | 2 |
| 6W4D | 6 | 2,4-D | 4 |
| 6W8D | 6 | 2,4-D | 8 |
| 6W2P | 6 | Picloram | 2 |
| 6W4P | 6 | Picloram | 4 |
| 6W8P | 6 | Picloram | 8 |

### 2.2.2. Transformation studies

*pAHC25* was transferred to 3 weeks old mature embryo derived callus on callus induction medium by Obitek-Biolab Gene Transfer System.

#### 2.2.2.1. Bacterial growth and plasmid isolation

*Escherichia coli* DH5α strain including *pAHC25* was grown in liquid Luria Broth medium supplemented with 50 mg/L ampicillin. After 37°C overnight incubation, 0.1 ml bacterial culture was inoculated Luria Broth agar medium supplemented with 50 mg/L ampicillin. Single colonies were selected and inoculated 50 ml Luria Broth medium with 50 mg/L ampicillin. After 37 °C overnight incubation, plasmid isolation was performed according to Qiagen Midi Kit procedure. After plasmid isolation, *pAHC25* was cut with *SmaI* and *SacI* restriction sites to confirm plasmid.

#### 2.2.2.2. Preperation of explants

14 days old mature embryo derived calli incubated in 2 mg/l 2,4-D medium were transferred to including 0.2 *M* mannitol medium for osmotic treatment.4 h pre- and 16 h post- bombardment.

#### 2.2.2.3. Transformation procedure

The laminar flow and inside of bombardment system was sterilized 70 % ethanol and steal meshes with fire. 60 mg of 1µm gold particles were surface sterilized washing with ethanol two times. After sterilization, they resuspended in 1 ml setrile distilled water. The suspension was allocated eppendorf tubes 30 µl with well vortex. 20 µl of sterile distilled water was added each aliquot. After that, 5 µl of the plasmid DNA having 1 µg/ µl concentration was put into the suspension. 50 µl of $CaCl_2$ and 20 µl of spermidine were quickly added the loading suspension and incubated on ice for 15 minutes. At the end of the incubation, the solution was centrifuged, discarded

supernatant and resuspended in 100 % 250 µl of ethanol. After washing step, solution was resuspended in 100 µl of 100 % ethanol. After good vortexing, 8 µl of suspension was loaded loading unit and bombarded the plant tissues using different conditions.

In this study, firstly, the gold particles were bombarded two different distances of 8 and 10 cm and four different helium gas pressures 30, 35, 40 and 45 bar using old loading unit. After gus results, transformation efficiency was calculated. It was chosen that 8 cm as distance and 30 and 35 bar as gas pressure to use for modified loading unit. The old loading unit terminates a narrow exit and this increases the affiliation or clamps of DNA coated gold particles. It is considered that clamps of gold particles causes detrimental effect for cells. The aluminium foil carrying gold particles used old unit also causes same results because of its narrow semi-diameter. The modified loading unit has wider exit and semi-diameter of aluminium foil than old one. It is considered that the modified system overcomes these limitations. The Figure 2.5 shows the old and modified loading systems.

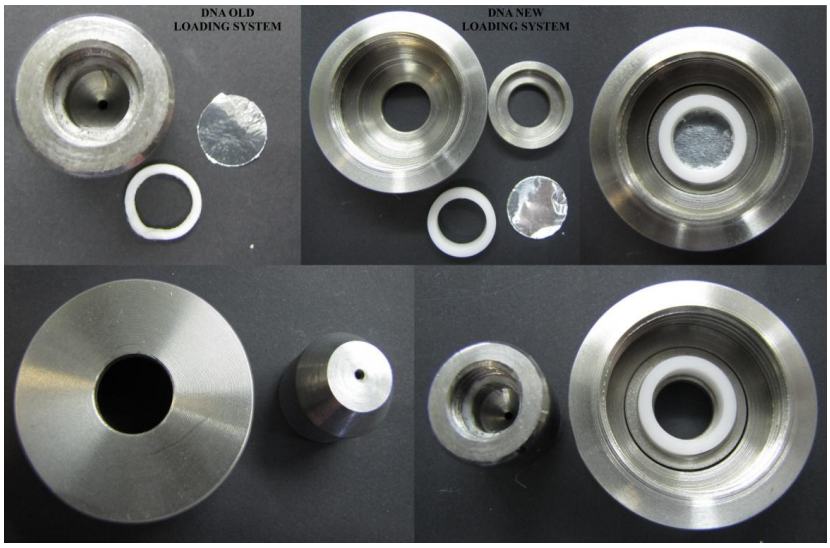


**Figure 2. 5.** The old and the modified gold loading units

#### 2.2.2.4. Histochemical GUS assay

Histochemical GUS assay was performed to determine transient gene expression. Bombarded explants were removed from incubation medium 2 days after bombardment. The mature embryo derived calli were treated with GUS solution preperared according to Jefferson (1987) in gus tubes overnight at 37ºC in dark. After incubation, the number of explants expressed gene and blue points were counted under stereomicroscope.

### 2.2.3. Statistical analyses

All statistical analyses were performed by using Minitab Statistical Software 13.0. One-Way (Unstucked) and Two-Way Analyses of variance (ANOVA) were used correlation between one or more dependent and independent variables.

# CHAPTER 3

# RESULTS AND DISCUSSION

## 3.1.Tissue culture studies

In this study, mature embryo derived regeneration of two wheat was evaluated together with three different concentration and exposure time of hormones 2,4-D and picloram. The callus induction, embryogenic callus induction and regeneration capacity of cultivars were discussed in the following sections.

### 3.1.1. Primary callus induction

Callus initiation was observed after 48-72 hours from all induction media for two cultivars. Mature embryo derived calli were incubated on callus induction medium to determine average callus fresh weight and primary callus induction rate. The time of initiation of callus was similar with other studies. It was reported 3 days later by Mendoza and Kaeppler in 2002, 2-3 days later by Chen et.al, in 2006, 2 days later by Bi and Wang in 2008 and 3 days later by Yu et.al, in 2008. For both cultivars, 4 and 6 weeks dark incubated calli were evaluated.

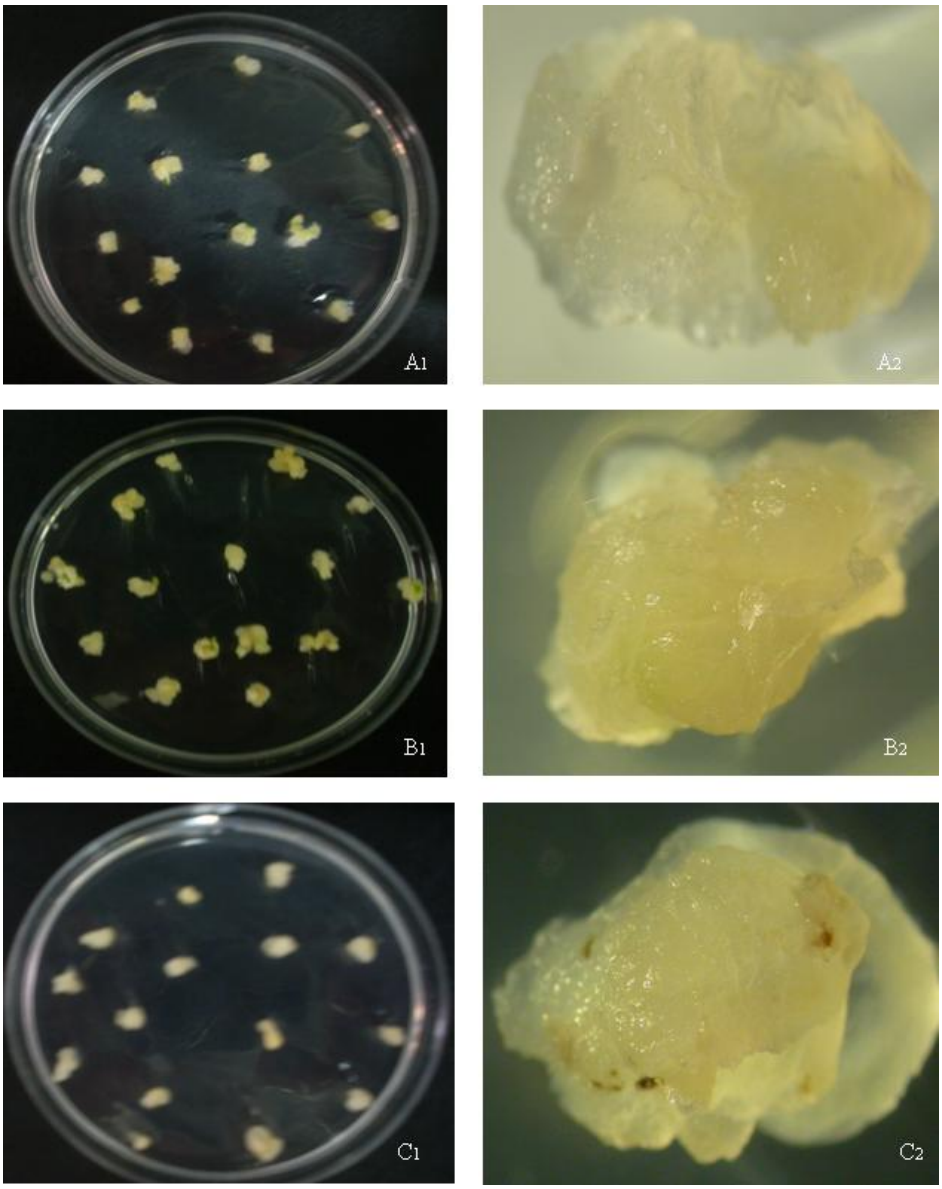


Figure 3. 1. Mirzabey calli from callus induction medium including 2,4-D. $A_1$ and $A_2$ were 3 weeks old calli and $B_1$ and $B_2$ were 5 weeks old calli in 2 mg/l 2,4-D medium. $C_1$ and $C_2$ were 5 weeks old calli in 8 mg/l 2,4-D.

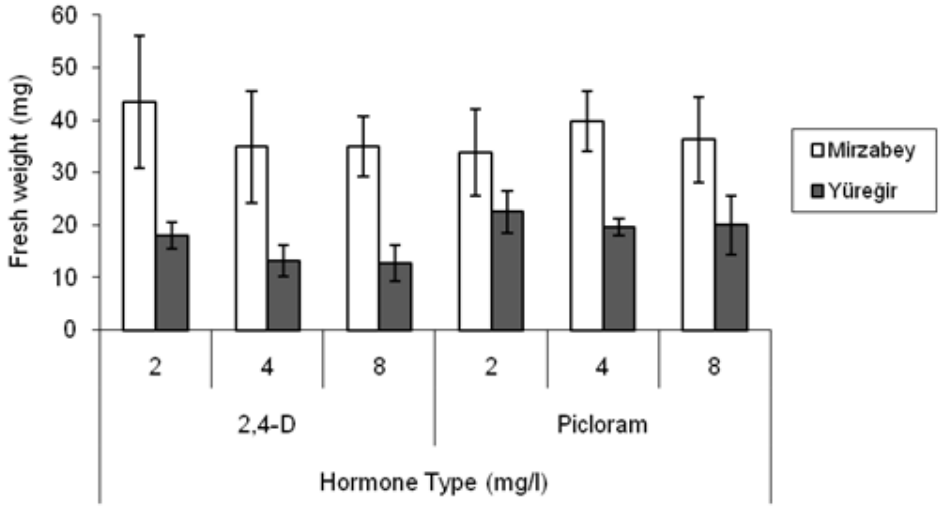


Figure 3. 2. Average fersh weight of 4 weeks old calli

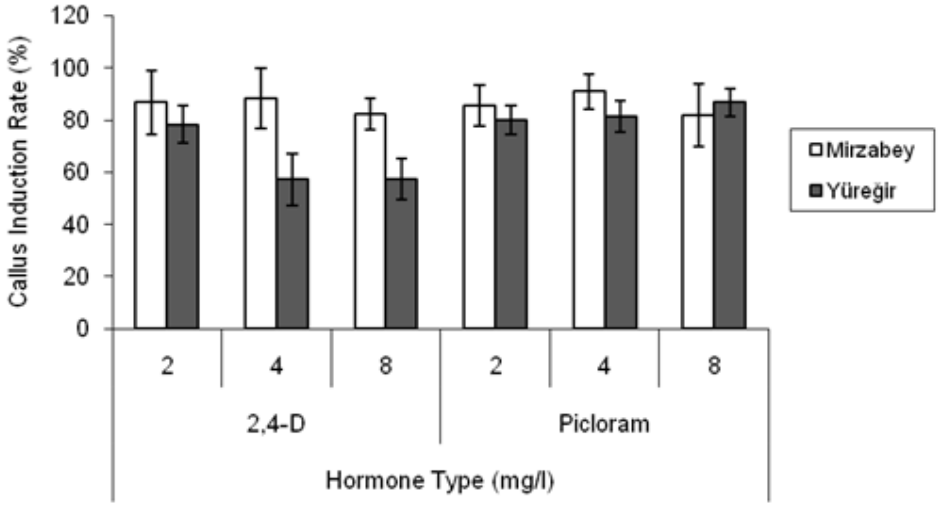


Figure 3. 3. Primary callus induction rate of 4 weeks old calli

Table 3. 1. Mirzabey callus fresh weight and primary callus induction rate after 4 weeks dark incubation.

| Factor[1] | | | Average callus | Primary callus |
|---|---|---|---|---|
| a | b | c | fresh weight (mg)[2] | induction rate (%)[3] |
| 4w | 2 | 2,4-D | 43.38 ± 12.57 | 86.75 ± 12.27 ab |
| 4w | 4 | 2,4-D | 34.88 ± 10.58 | 88.25 ± 11.54 ab |
| 4w | 8 | 2,4-D | 35.00 ± 5.73 | 82.38 ± 6.07 b |
| 4w | 2 | Picloram | 33.83 ± 8.28 | 85.67 ± 7.82 ab |
| 4w | 4 | Picloram | 39.83 ± 5.74 | 91.00 ± 6.78 a |
| 4w | 8 | Picloram | 36.29 ± 8.16 | 81.86 ± 11.98b |

Means denoted by different letters in a column are significantly different at $P < 0.05$ according to One-way ANOVA test. [1]Factor a: dark incubation time; Factor b: hormone concentration mg/l; Factor c: hormone type. [2]Weight of calli/No.cultured mature embryo. [3]No. explants forming callus/Cultured embryo X 100.

Figure 3.1 shows Mirzabey calli from callus induction medium including 2,4-D. According to Figure 3.2 and Table 3.1, 4 weeks old dark incubated mature embryo derived Mirzabey callus gave the highest average fresh weight of callus from 4W2D medium. Figure 3.2 shows Mirzabey Calli from induction medium including 2,4-D.

However, there was no significantly differences including other concentration of 2,4-D and picloram. It was observed including 4W4P medium had the highest callus induction frequency (91 %). 4W8D medium primary callus induction rate was the minimum (82,38 %), (Figure 3.3).

Table 3. 2.Yüreğir callus fresh weight and primary callus induction rate after 4 weeks dark incubation

| Factor[1] | | | Average callus fresh weight (mg)[2] | Primary callus induction rate (%)[3] |
|---|---|---|---|---|
| a | b | c | | |
| 4w | 2 | 2,4-D | 18.000 ± 2.619 b | 78.375 ± 7.009 a |
| 4w | 4 | 2,4-D | 13.200 ± 3.033 c | 57.200 ± 9.960 b |
| 4w | 8 | 2,4-D | 12.800 ± 3.421 c | 57.400 ± 7.635 b |
| 4w | 2 | Picloram | 22.500 ± 4.041 a | 80.000 ± 5.715 a |
| 4w | 4 | Picloram | 19.600 ± 1.517 ab | 81.400 ± 5.857 a |
| 4w | 8 | Picloram | 20.000 ± 5.598 ab | 86.750 ± 5.315 a |

Means denoted by different letters in a column are significantly different at $P < 0.05$ according to One-way ANOVA test. [1]Factor a: dark incubation time; Factor b: hormone concentration mg/l; Factor c: hormone type. [2]Weight of calli/No.Cultured mature embryo. [3]No. explants forming callus/Cultured embryo X 100.

According to Figure 3.2 and Table 3.2, 4 weeks dark incubated Yüreğir calli gave the highest average callus fresh weight in 4W2P medium (22.50 mg) and the lowest in 4W8D medium (12.80 mg).The primary callus induction rate was the maximum in 4W8P medium (86.75 %) and the minimum in 4W4D medium (57.2 %), Figure 3.3.

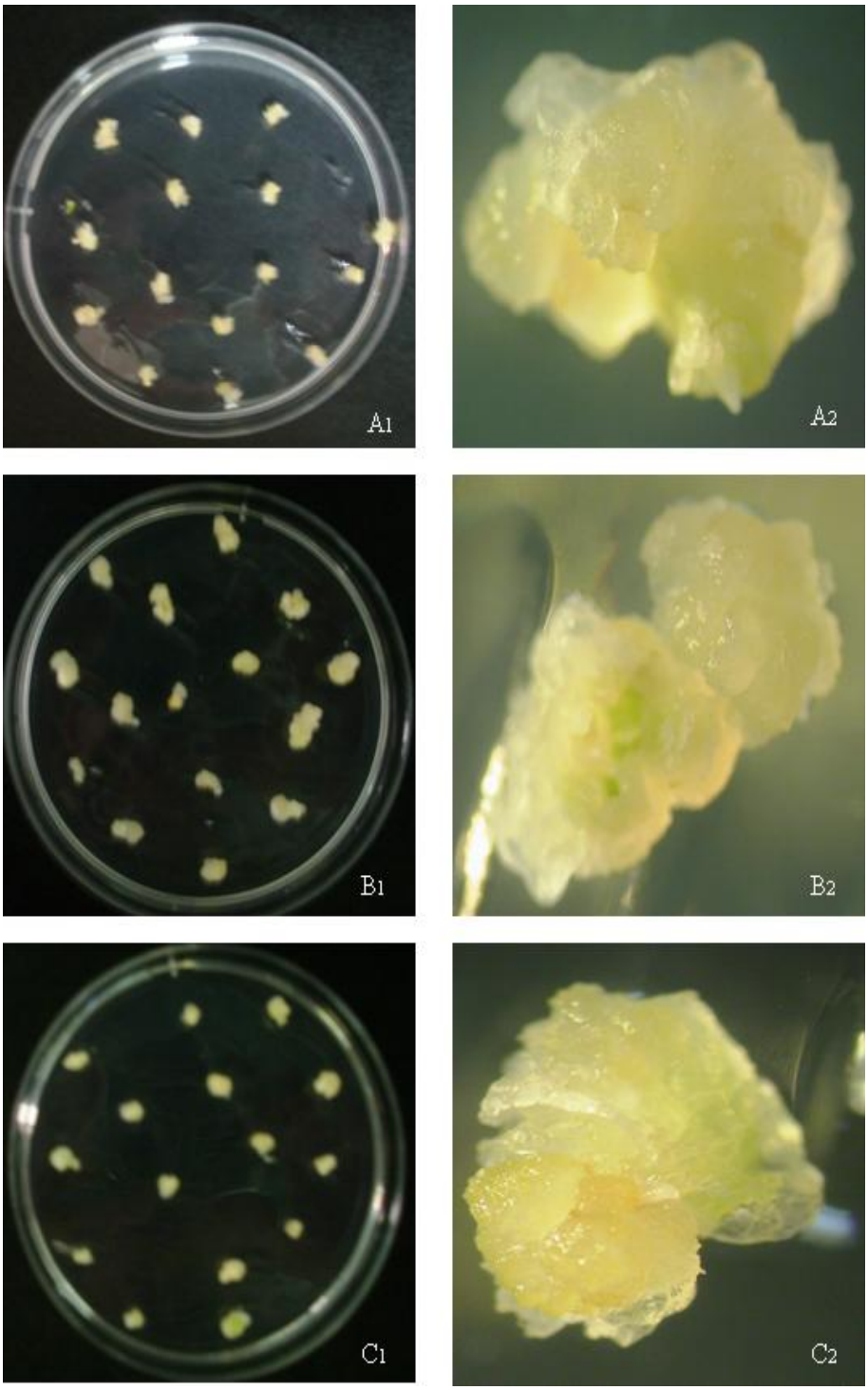


Figure 3. 4. Mirzabey calli from callus induction medium including picloram. $A_1$ and $A_2$ were 3 weeks old calli and $B_1$ and $B_2$ were 5 weeks old calli in 2 mg/l picloram medium. $C_1$ and $C_2$ were 5 weeks old calli in 4 mg/l picloram.

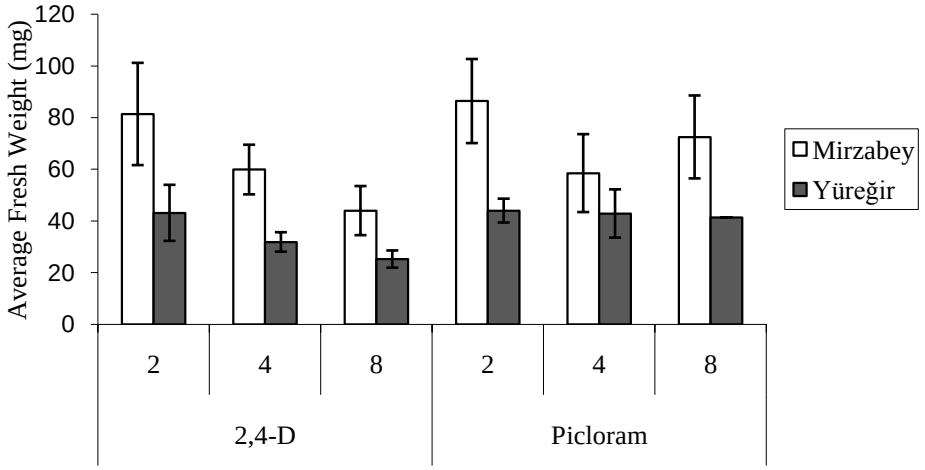


Figure 3. 5. Average fresh weight of 6 weeks old calli

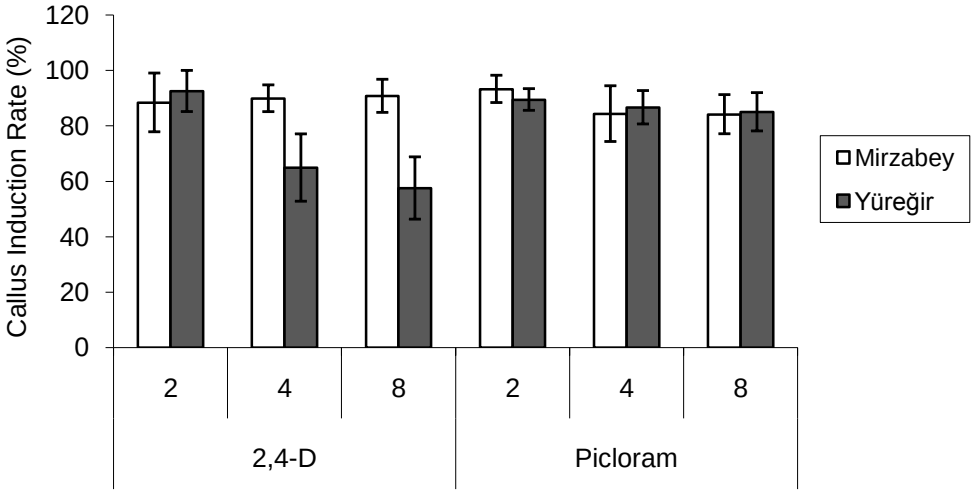


Figure 3. 6. Primary callus induction rate of 6 weeks old calli

Table 3. 3. 6 weeks old Mirzabey callus fresh weight and primary callus induction rate

| Factor[1] | | | Average callus fresh weight[2] (mg) | Primary callus induction rate[3] (%) |
|---|---|---|---|---|
| a | b | c | | |
| 6w | 2 | 2,4-D | 81.38 ± 19.78 a | 88.375 ± 10.596 ab |
| 6w | 4 | 2,4-D | 59.88 ± 9.60 b | 89.875 ± 4.824 ab |
| 6w | 8 | 2,4-D | 44.00 ± 9.50 c | 90.750 ± 5.970 ab |
| 6w | 2 | Picloram | 86.38 ± 16.27 a | 93.250 ± 4.921 a |
| 6w | 4 | Picloram | 58.50 ± 15.08 b | 84.333 ± 10.053 b |
| 6w | 8 | Picloram | 72.50 ± 16.04 ab | 84.125 ± 7.060 b |

Means denoted by different letters in a column are significantly different at $P < 0.05$ according to One-way ANOVA test. [1]Factor a: dark incubation time; Factor b: hormone concentration mg/l; Factor c: hormone type. [2]Weight of calli/No.cultured mature embryo. [3]No. explants forming callus/Cultured embryo X 100.

Figure 3.4 shows Mirzabey calli from callus induction medium including picloram. Figure 3.5 and 3.6 and Table 3.3 show average callus fresh weight and callus induction rate of 6 weeks dark incubated Mirzabey mature embryo derived callus. Average callus fresh weight (86.38 mg) was the highest in 6W2P medium (Figure 3.5). Also, 6W2D medium was significantly different than other used hormone concentration according to average fresh weight. The minimum average fresh weight was 44.00 mg in 6W8D medium. According to primary callus induction rate, the maximum rate (93.25 %) was in 6W2P medium (Figure 3.6). There was no significantly difference other auxin hormone concentration.

Table 3. 4.Yüreğir callus fresh weight and primary callus induction rate after 6 weeks dark incubation

| Factor1 | | | Average callus fresh weight (mg)[2] | Primary callus induction rate (%)[3] |
|---|---|---|---|---|
| a | b | c | | |
| 6w | 2 | 2,4-D | 43.125 ± 10.842 a | 92.500± 7.426 a |
| 6w | 4 | 2,4-D | 31.875 ± 3.758 b | 64.875 ± 12.147 b |
| 6w | 8 | 2,4-D | 25.250 ± 3.327 c | 57.500 ± 11.238 b |
| 6w | 2 | Picloram | 44.000 ± 4.619 a | 89.429 ± 3.910 a |
| 6w | 4 | Picloram | 42.875 ± 9.342 a | 86.625 ± 6.022 a |
| 6w | 8 | Picloram | 41.375 ±4 .406 a | 85.000 ± 6.908 a |

Means denoted by different letters in a column are significantly different at $P < 0.05$ according to One-way ANOVA test. [1]Factor a: dark incubation time; Factor b: hormone concentration mg/l; Factor c: hormone type. [2]Weight of calli/Cultured mature embryo. [3]No. explants forming callus/Cultured embryo X 100.

When 6 weeks dark incubated Yüreğir mature embryo derived calli were evaluated, 6W2P medium had the 44.00 mg average callus fresh weight (Figure 3.5 and Table 3.4). Other auxin hormone concentration used for this study except 4 mg/l and 8 mg/l 2,4-D did not give different response according to statistical analyses. Average primary callus induction rate was the highest in 6W2D medium (92.5 %) according to Figure 3.6 and Table 3.4.

Table 3. 5. Two-Way ANOVA analysis of 4 and 6 weeks old Mirzabey and Yüreğir average callus fresh weight

| Dependent variables | | Independent variables | | |
|---|---|---|---|---|
| | Dark Period | [1]H | [2]C | [3]HxC |
| Mirzabey | 4w | 0.16 | 0.39 | 2.39 |
| | 6w | 5.92* | 15.00*** | 4.30* |
| Yüreğir | 4w | 23.56*** | 4.45* | 0.42 |
| | 6w | 22.34*** | 8.97*** | 5.06** |

[1]Hormone type; [2]Concentration; [3]Hormone type X Concentration, *p<0.05; **p<0.01; ***p<0.001

When the Table 3.5. was evaluated, auxin types and their concentrations had significance effects on average callus fresh weight of cultivars. 6 weeks dark incubated Mirzabey callus fresh weight was significantly dependent hormone type ($p<0.05$), hormone concentration ($p<0.001$) and hormone type and concentration ($p<0.05$). However, 4 weeks dark incubated Mirzabey callus fresh weight did not show significant differences according to independent variables. 6 weeks dark incubated Yüreğir callus fresh weight was also significantly dependent hormone type ($p<0.001$), hormone concentration ($p<0.001$) and hormone type and concentration ($p<0.01$). 4 weeks dark incubated Yüreğir average callus fresh weight was significantly affected hormone type ($p<0.001$) and concentration ($p<0.05$).

Wei and colleagues observed callus fresh weight of ten different spring wheat variable value between 44.2 and 153.4 mg. They suggested the cultivar genotype significantly affected callus fresh weight. In our study, cultivars gave different response among same conditions such as 4W2D Yüreğir was 18 mg and Mirzabey 43.38 mg. Mendoza and Kaeppler reported fresh weight of Bobwhite a model cultivar in tissue culture for including 2 mg/l 2,4-D between 133-281 mg, 4 mg/l 2,4-

D 30-86 mg, 2 mg/l picloram 499-607 mg and 4 mg/l picloram 405-565 mg. They also reported auxin type and concentration had significantly effect on callus fresh weight. This approach has similar results in this study. Hormone type and concentration significantly affected Mirzabey 6 weeks old and Yüreğir 4 and 6 weeks old average callus fresh weight.

Table 3. 6. Two-Way ANOVA analysis primary callus induction rate

| Dependent variables Dark Period | | Independent variables [1]H | [2]C | [3]HxC |
|---|---|---|---|---|
| Mirzabey | 4w | 0.02 | 2.06 | 0.15 |
| | 6w | 1.21 | 1.17 | 2.85 |
| Yüreğir | 4w | 48.02*** | 5.20* | 10.59*** |
| | 6w | 38.18*** | 22.46*** | 13.88*** |

[1]Hormone type; [2]Concentration; [3]Hormone type X Concentration, *p<0.05; **p<0.01; ***p<0.001

According to Table 3.6, it was not observed significantly correlation between primary callus induction rate and independent variables for Mirzabey 4 weeks and 6 weeks dark incubated calli. However, primary callus induction rate was significantly dependent on hormone type, concentration and hormone type together with concentration for Yüreğir 4 weeks and 6 weeks dark incubated calli.

In this study, the highest primary callus induction rate for Mirzabey was in 6W2P (93.25 %) and the lowest was in 4W8D medium. For Yüreğir, the maximum rate was in 6W2D and the minimum was in 4W4D medium. The primary callus induction rate was found variable rate in different studies. Bi and Wang (2008) found between

68.75-96.20 % in 2 mg/l 2,4-D from four wheat mature embryo based calli. The callus induction frequency was recorded between 11.6-89.6 % including 8 mg/l 2,4-D medium (Chen et.al, 2006). In this study, callus induction rate in 8 mg/l 2,4-D varied between 57.40-90.75 %. Chauhan and colleagues (2007) used 2 mg /l 2,4-D and found primary callus formation rate between 82-85% for *T.aestivum* and 77-79 % for *T.durum* 2 mg/l 2,4-D in medium being critical point for callus induction was used by Yu et.al, in 2008 and the callus induction rate was between 70.8-95 %. Bi and colleagues (2007) reported the primary callus formation rate was 86.67 % in including 2 mg/l 2,4-D, 87.86 % in 3 mg/l 2,4-D and 85% in 4 mg/l 2,4-D. They also claimed the necessary of 2 mg/l of 2,4-D in medium and the quality of callus, hormone type, concentration and genotype had significantly effects on primary callus induction rate. Wei and colleagues (2003) also observed auxin concentration and genotype significantly affected primary callus induction rate changing between 6.9-82 %. According to results, it was observed Yüreğir primary callus induction rate was significantly affected by hormone type and concentration. Mirzabey calli did not show significantly difference.

### 3.1.2. Embryogenic callus induction

Primary callus were transferred to embryogenic callus induction medium including same hormone type and concentration as callus induction medium. However, BA and NAA were added to media to increase embryogenic callus formation. Embryogenic and non-embryogenic callus structures were observed. While embryogenic calli were pale, smooth, compact, regenerable and contained embryogenic structure, non-embryogenic calli were like a cream color, soft and watery. The globular and heart shape green spots were demonstrated in Figure 3.7 for Mirzabey and Figure 3.11 for Yüreğir. After 4 weeks embryogenic callus induction medium incubation, callus fresh weight and embryogenic callus formation rate were evaluated.

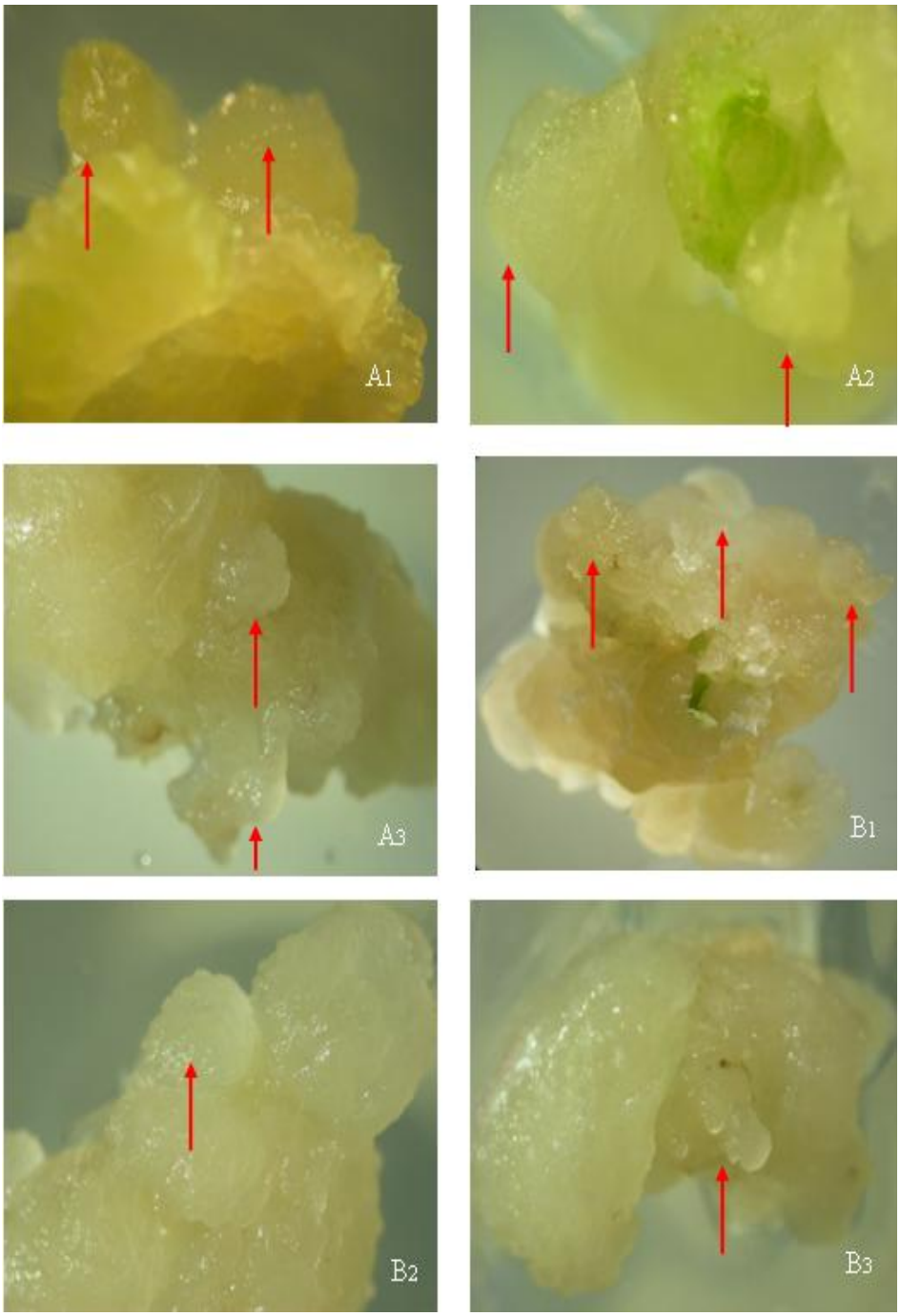


Figure 3. 7.Embryogenic calli structures of Mirzabey. Red arrows show globular or heart shape embryogenic sites of callus. $A_1$ from 7 weeks old, $A_2$ from 8 weeks old and $A_3$ from 9 weeks old incubated in 2 mg/l 2,4-D. $B_1$ from 10 weeks old in 2 mg/l picloram, $B_2$ from 9 weeks old in 4 mg/l picloram and $B_3$ from 8 weeks old in 8 mg/l picloram.

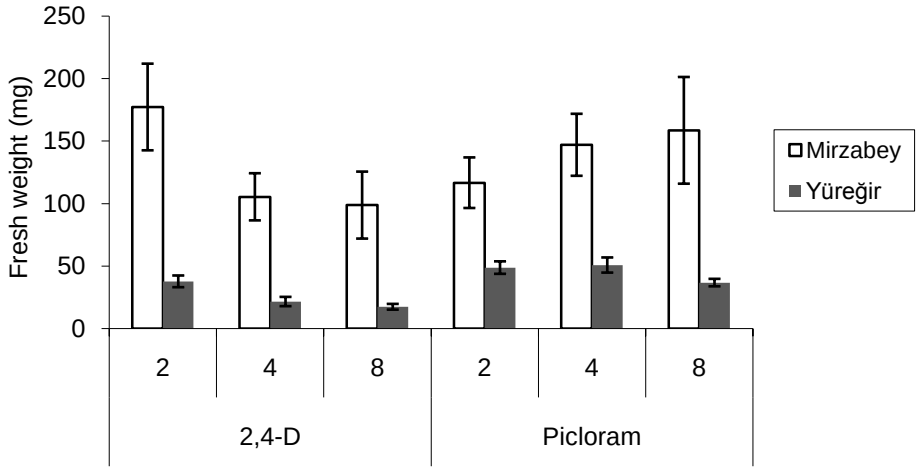


Figure 3. 8.Average fresh weight of 8 weeks old calli

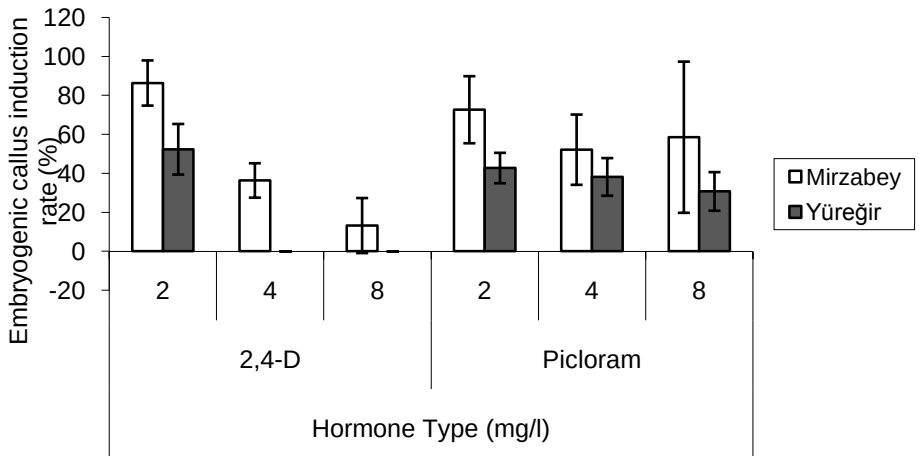


Figure 3. 9.Embryogenic callus induction rate of 8 weeks old calli

Table 3. 7. 8 weeks old Mirzabey callus fresh weight and embryogenic callus induction rate

| Factor[1] | | | Average callus fresh weight (mg)[2] | Embryogenic callus induction rate (%)[3] |
|---|---|---|---|---|
| a | b | c | | |
| 4w | 2 | 2,4-D | 177.25 ± 34.67 a | 86.38 ± 11.58 a |
| 4w | 4 | 2,4-D | 105.38 ± 18.86 c | 36.38 ± 8.83 c |
| 4w | 8 | 2,4-D | 98.75 ± 26.80 c | 13.25 ± 14.12 d |
| 4w | 2 | Picloram | 116.67 ± 20.23 bc | 72.67 ± 17.20 ab |
| 4w | 4 | Picloram | 147.00 ± 24.81 a | 52.17 ± 18.02 bc |
| 4w | 8 | Picloram | 158.57 ± 42.69 ab | 58.57 ± 38.78 abc |

Means denoted by different letters in a column are significantly different at $P < 0.05$ according to One-Way ANOVA test. [1]Factor a: dark incubation time; Factor b: hormone concentration mg/l; Factor c: hormone type. [2]Weight of calli/No.Primary calli transferred onto embryogenic callus induction medium. [3]No. explants forming callus/ No.Primary calli transferred onto embryogenic callus induction medium X 100.

According to Figure 3.8 and Table 3.7, 4W2D and 4W4P media gave the significantly difference results in terms of average callus fresh weight. The weight was 177.25 mg for 4W2D and 147.00 mg for 4W2P. The minimum weight was 98.75 for 4W8D medium. 8 weeks old mirzabey calli 4 weeks dark incubated embryogenic callus induction rate was evaluated, the significance difference from other media was observed in 4W2D medium and rate was 86.38 %, Figure 3.9. The minimum embryogenic callus induction rate was 13.25 % in 4W8D medium. The necrotic tissues were observed in high concentrations of 2,4-D, Figure 3.10.

Table 3. 8. 8 weeks old Yüreğir callus fresh weight and embryogenic callus induction rate

| Factor[1] | | | Average callus fresh weight (mg)[2] | Embryogenic callus induction rate (%)[3] |
|---|---|---|---|---|
| a | b | c | | |
| 4w | 2 | 2,4-D | 37.75 ± 4.683 b | 52.38 ± 12.983 a |
| 4w | 4 | 2,4-D | 21.60 ± 3.715 c | 0.000 |
| 4w | 8 | 2,4-D | 17.40 ± 2.302 c | 0.000 |
| 4w | 2 | Picloram | 48.75 ± 4.992 a | 42.75 ± 7.805 ab |
| 4w | 4 | Picloram | 50.80 ± 6.058 a | 38.20 ± 9.654 ab |
| 4w | 8 | Picloram | 36.75 ± 2.986 b | 30.75 ± 9.912 b |

Means denoted by different letters in a column are significantly different at $P < 0.05$ according to One-Way ANOVA test. [1]Factor a: dark incubation time; Factor b: hormone concentration mg/l; Factor c: hormone type. [2]Weight of calli/No.Primary calli transferred onto embryogenic callus induction medium. [3]No. explants forming callus/ No.Primary calli transferred onto embryogenic callus induction medium X 100.

According to Figure 3.8 and Table 3.8, 8 weeks old Yüreğir mature embryo based average callus fresh weight was the maximum in 4W4P medium (50.80 mg). However, there was no significantly difference between 4W4P and 4W2P media. The lowest fresh weight was in 4W8D medium (17.40 mg). While the highest embryogenic callus formation rate was observed in 4W2D medium, this formation was not observed in 4W4D and 4W8D media, Figure 3.9. Browning cells were observed for these two medium (Figure 3.10).

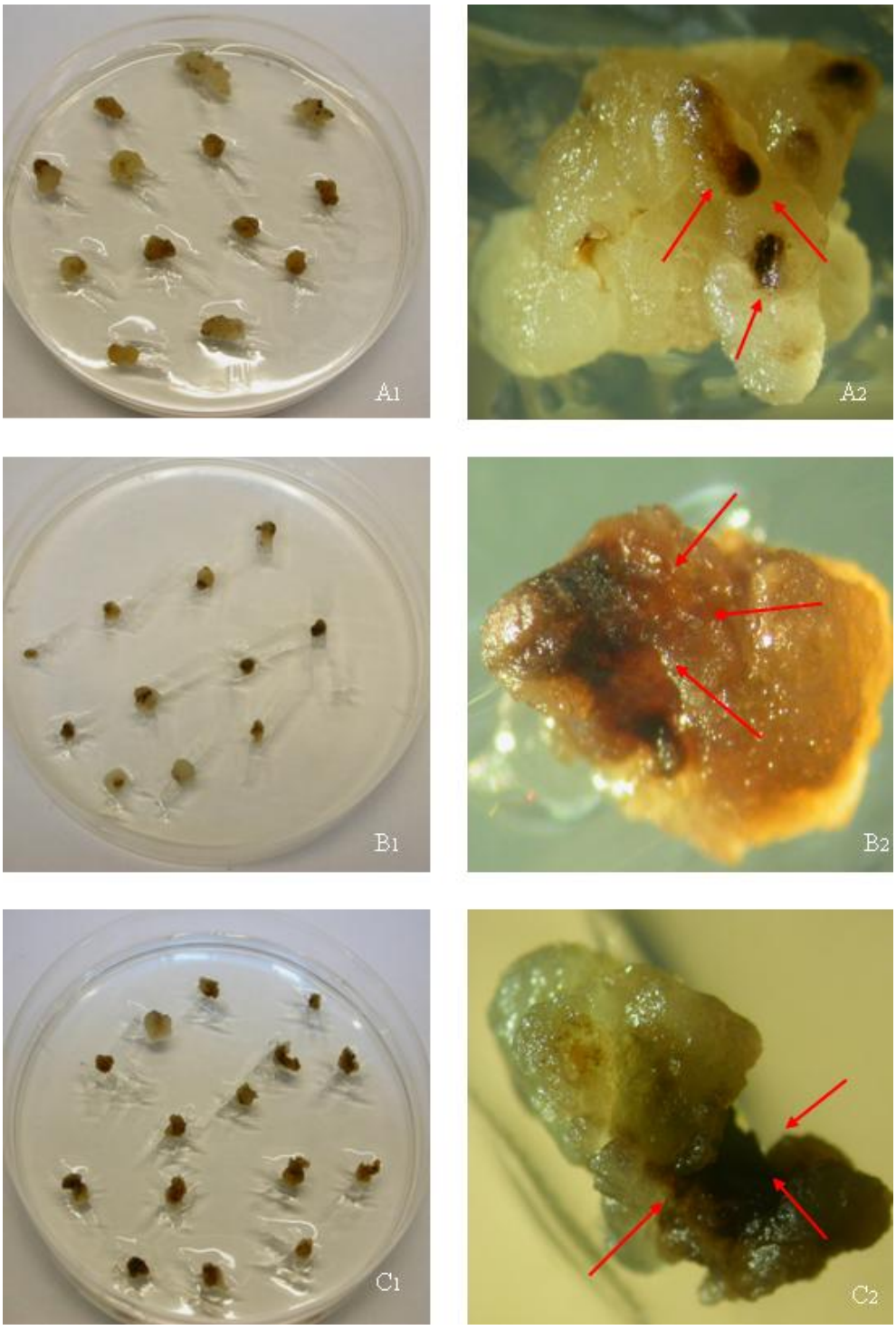


Figure 3. 10.Necrotic tissues from high concentration of 2,4-D. Red arrows show necrotic areas of callus. $A_1$ and $A_2$ from 9 weeks old Mirzabey culture in 8 mg/l, $B_1$ and $B_2$ from 8 weeks old Yüreğir culture in 4 mg/l, $C_1$and $C_2$ from 8 weeks old Yüreğir culture in 8 mg/l.

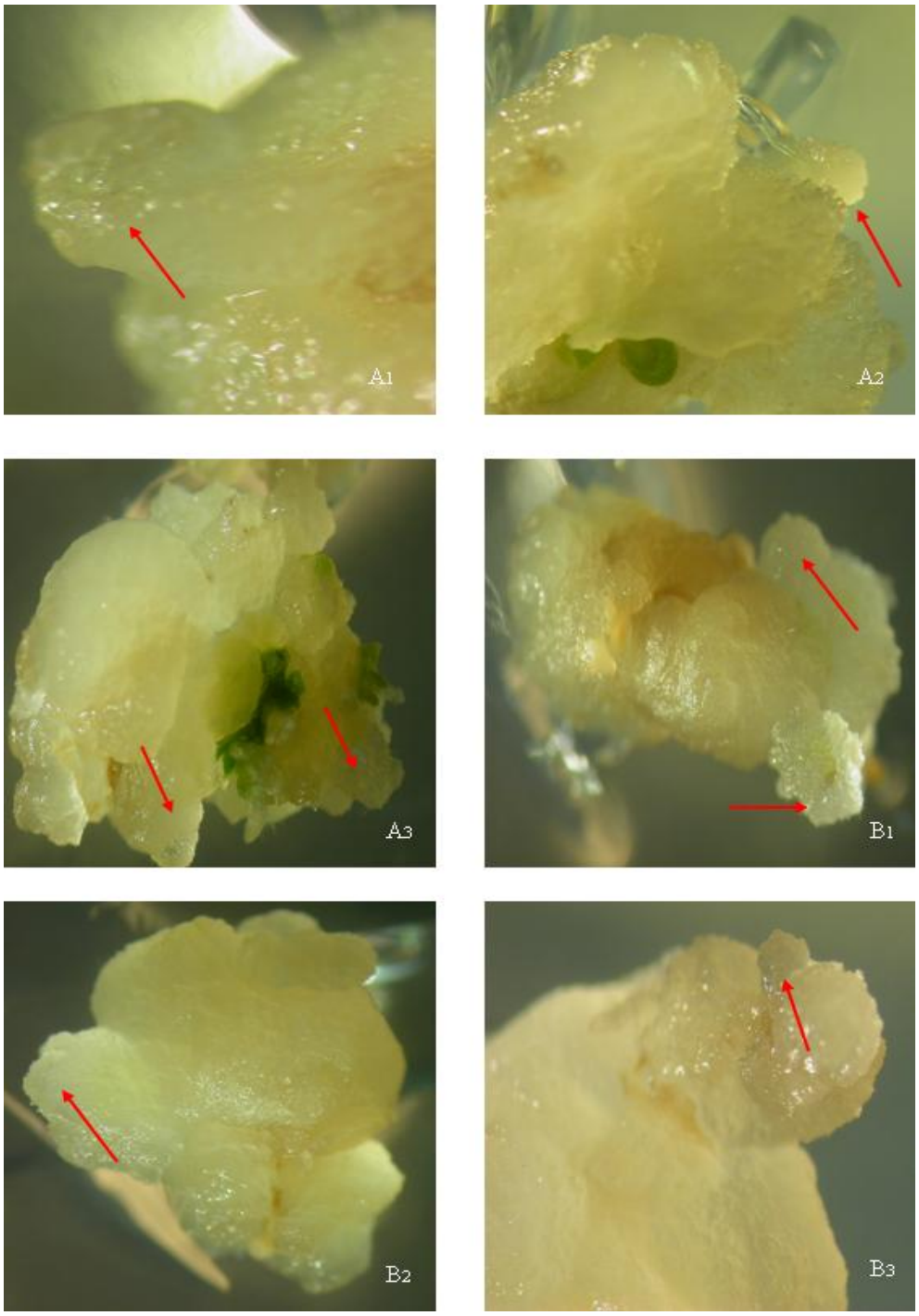


Figure 3. 11.Embryogenic calli structures of Yüreğir. Red arrows show globular or heart shape embryogenic sites of callus. $A_1$ from 7 weeks old, $A_2$ from 8 weeks old and $A_3$ from 9 weeks old incubated in 2 mg/l 2,4-D. $B_1$ from 10 weeks old in 2 mg/l picloram, $B_2$ from 9 weeks old in 4 mg/l picloram and $B_3$ from 8 weeks old in 8 mg/l picloram.

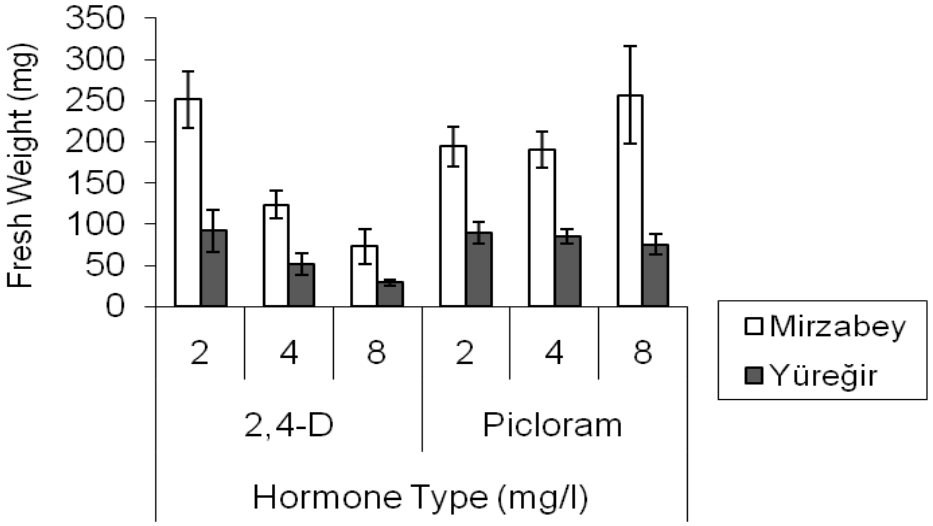


Figure 3. 12. Average fresh weight of 10 weeks old calli

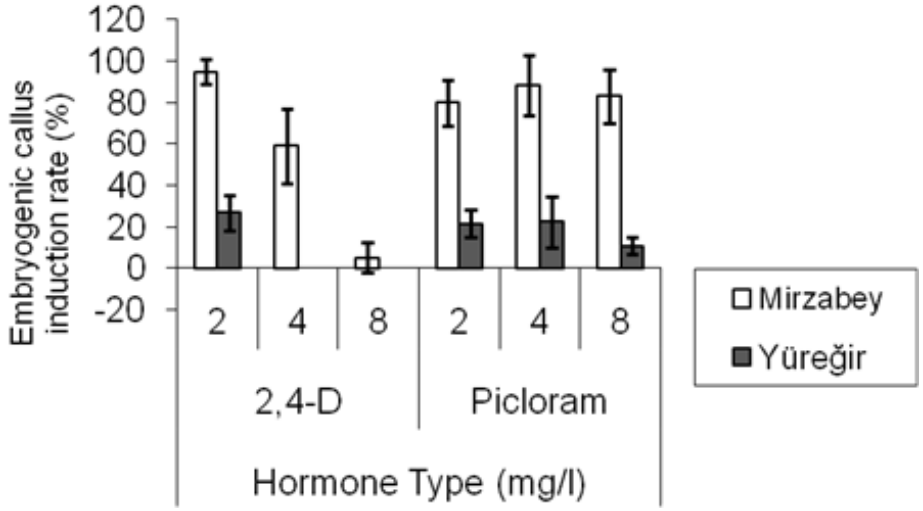


Figure 3. 13. Embryogenic callus induction rate of 10 weeks old calli

Table 3. 9. 10 weeks old Mirzabey callus fresh weight and embryogenic callus induction rate.

| Factor[1] | | | Average callus fresh weight (mg)[2] | Embryogenic callus induction rate (%)[3] |
|---|---|---|---|---|
| a | b | c | | |
| 6w | 2 | 2,4-D | 251.38 ± 34.60 a | 94.88 ± 6.24 a |
| 6w | 4 | 2,4-D | 123.50 ± 16.59 c | 59.00 ± 17.81 c |
| 6w | 8 | 2,4-D | 72.75 ± 21.03 d | 5.13 ± 7.10 d |
| 6w | 2 | Picloram | 194.38 ± 23.75 b | 79.88 ± 11.23 b |
| 6w | 4 | Picloram | 190.17 ± 21.93 b | 88.17 ± 14.43 ab |
| 6w | 8 | Picloram | 256.75 ± 58.99 a | 83.00 ± 12.86 b |

Means denoted by different letters in a column are significantly different at $P < 0.05$ according to One-Way ANOVA test. [1]Factor a: dark incubation time; Factor b: hormone concentration mg/l; Factor c: hormone type. [2]Weight of calli/No.Primary calli transferred onto embryogenic callus induction medium. [3]No. explants forming callus/ No.Primary calli transferred onto embryogenic callus induction medium X 100.

If the Figure 3.12 and Table 3.9 were evaluated, callus average fresh weight was the highest in 6W8P medium (256.75 mg). There was no significantly difference between 6W8P and 6W2D media according to fresh weight. These two media were signicantly difference other four media. When the embryogenic callus induction rate was evaluated, 6W2D medium had the maximum rate (94.88 %), Figure 3.13. The minimum rate of embryogenic callus induction was observed in 6W8D medium (5.13 %).

Table 3. 10. 10 weeks old Yüreğir callus fresh weight and embryogenic callus induction rate

| Factor[1] | | | Average callus fresh weight (mg)[2] | Embryogenic callus induction rate (%)[3] |
|---|---|---|---|---|
| a | b | c | | |
| 6w | 2 | 2,4-D | 91.75 ± 25.52 a | 26.875 ± 8.408 a |
| 6w | 4 | 2,4-D | 51.38 ± 12.86 b | 0.000 |
| 6w | 8 | 2,4-D | 29.13 ± 3.31 c | 0.000 |
| 6w | 2 | Picloram | 89.57 ± 12.79 a | 21.429 ± 6.630 a |
| w | 4 | Picloram | 84.88 ± 8.59 a | 22.375 ± 12.130 a |
| 6w | 8 | Picloram | 75.50 ± 12.38 a | 10.875 ± 4.051 b |

Means denoted by different letters in a column are significantly different at $P < 0.05$ according to One-Way ANOVA test. [1]Factor a: dark incubation time; Factor b: hormone concentration mg/l; Factor c: hormone type. [2]Weight of calli/No.Primary calli transferred onto embryogenic callus induction medium. [3]No. explants forming callus/ No.Primary calli transferred onto embryogenic callus induction medium X 100.

When 10 weeks old Yüreğir average callus fresh weight was evaluated, there was no significantly difference between 6W2D, 6W2P, 6W4P and 6W8P media (Figure 3.12 and Table 3.10). However, media 6W4D and 6W8D were significantly different from other media (Figure 3.12). The maximum average callus fresh weight was in 6W2D medium (91.75 mg) and the minimum average callus fresh weight was 6W8D medium (29.13 mg). Yüreğir embryogenic callus induction rate was lower than Mirzabey (Figure 3.13). The highest embryogenic callus induction rate was in 6W2D medium (26.875 %). The embryogenic callus formation was not determined in 6W4D and 6W8D media.

Table 3. 11. Two-Way ANOVA analysis of Mirzabey and Yüreğir average callus fresh weight after embryogenic callus induction medium

| Dependent variables | | Independent variables | | |
|---|---|---|---|---|
| | | [1]H | [2]C | [3]HxC |
| Mirzabey | 4w | 2.27 | 2.06 | 17.07*** |
| | 6w | 42.96*** | 18.20*** | 52.67*** |
| Yüreğir | 4w | 151.34*** | 33.17*** | 11.18*** |
| | 6w | 38.53*** | 28.03*** | 11.88*** |

[1]Hormone type; [2]Concentration; [3]Hormone type X Concentration, *p<0.05; **p<0.01; ***p<0.001

If Table 3.11. was analyzed, it was observed that average callus fresh weight of 8 weeks old Mirzabey calli 4 weeks dark incubated were not affected individually hormone type and hormone concentration. However, hormone type together with hormone concentration significantly affected 8 weeks old callus fresh weight ($p<0.001$). 10 weeks old Mirzabey calli 6 weeks dark incubated exhibited significant difference in terms of hormone type, concentration and hormone type together with concentration ($p<0.001$). Also, fresh weight of 8 and 10 weeks old Yüreğir mature embryo based calli were affected hormone type concentration and hormone type and together with concentration ($p<0.001$).

Table 3. 12. Two-Way ANOVA analysis embryogenic callus induction rate

| Dependent variables Dark Period | | Independent variables [1]H | [2]C | [3]HxC |
|---|---|---|---|---|
| Mirzabey | 4w | 72.21*** | 52.64*** | 58.25*** |
| | 6w | 6.51* | 18.56*** | 7.68** |
| Yüreğir | 4w | 35.38*** | 38.72*** | 21.01*** |
| | 6w | 21.69*** | 30.36*** | 16.20*** |

[1]Hormone type; [2]Concentration; [3]Hormone type X Concentration, *p<0.05; **p<0.01; ***p<0.001

When the Two-way ANOVA analysis of embryogenic callus induction rate in Table 3.12 was evaluated, hormone type, concentration and hormone type together with concentration significantly affected embryogenic capacity of both of cultivars.

The embryogenic callus formation rate for 2 mg/l 2,4-d 63.34%, 3 mg/l 28.34 % and 4 mg/l 18.44 was reported by Bi and colleagues in 2007. The embryogenic capacity was between 36.38-59 % in 4 mg/l 2,4-D medium for Mirzabey and no embryogenic formation for Yüreğir. Chen and colleagues (2006) reported the rate was between 49-83 % in including 8 mg/l 2,4-D. In this study, the embryogenic capacity was between 5.13-13.25 for Mirzabey in 8 mg/l 2,4-D. The embryogenic callus structure was not recorded for Yüreğir in same concentration. Mendoza and Kaeppler (2002) observed detrimental effect of high concentration of 2,4-D resulting brownish and necrotic appearance of 90 % calli. The necrotic tissues were shown in Figure 3.7. This structure was observed in this study for 4 mg/l and 8 mg/l 2,4-D concentration. Higher concentration of 2,4-D increases probably somatic mutation (Choi et.al, 2001). The embryogenic callus induction frequency was reported between 27.27-90 % in including 2 mg/l 2,4-D medium by Bi and Wang in 2008. Yu and colleagues (2008) reported the rate was between 30.9-48.5 % in 2mg/l 2,4-D. In this study, the embryogenic capacity was 94.88 % in 6W2D and 86.38 % in 4W2D for Mirzabey. For Yüreğir, rate was 26.875 % in 6W2D and 52.38 in 4W2D. The light of these information, hormone type, concentration and genotype affected embryogenic callus formation from mature embryo based callus culture. The results recorded in this study show similarity with literature.

### 3.1.3. Regeneration

Embryogenic calli being compact, pale color and nodular type were incubated in auxin hormone free regeneration medium. They were incubated on 3000 lux light and 16/8 photoperiod at 25ºC. After regeneration medium, regeneration rate and culture efficiency were evaluated. Shoot number and rooted calli were cıunted and average shoot number and root formation rate were determined. 8 cm regenerated planlets were transferred to greenhouse.

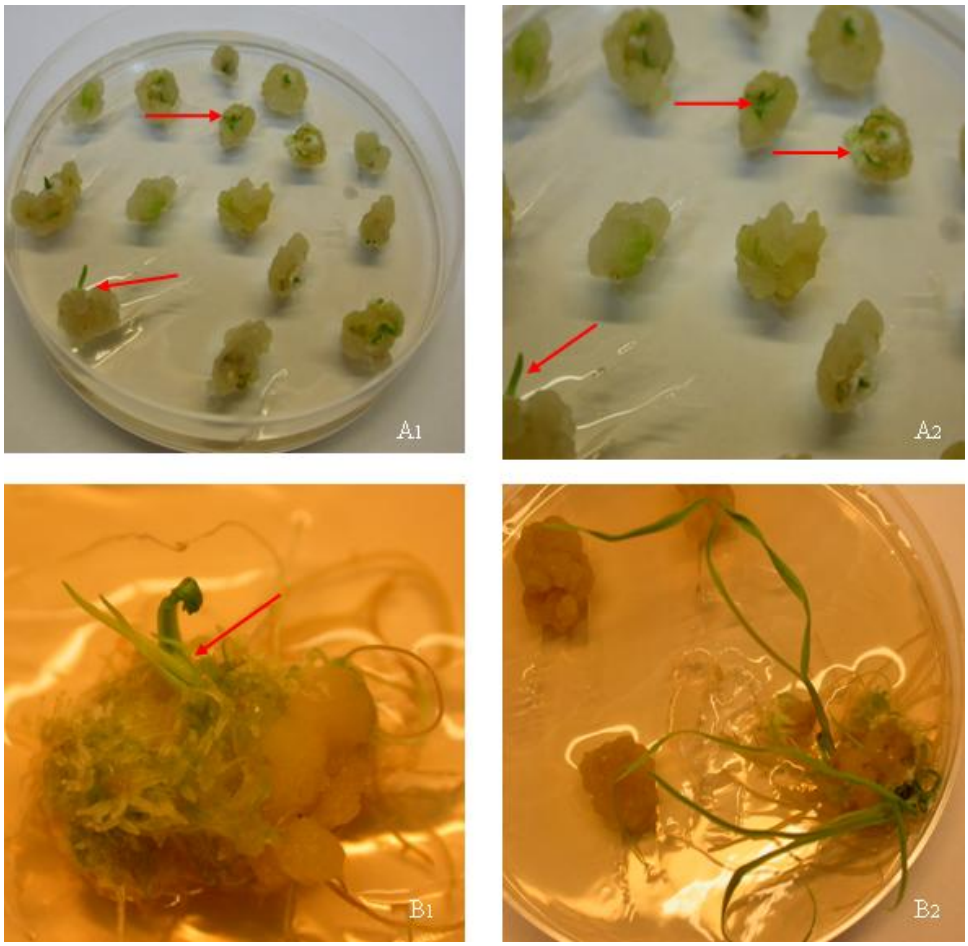


Figure 3. 14.Shoot formation and plant regeneration in regeneration medium. Red arrows show shoot initiation. $A_1$ and $A_2$ from 10 weeks old Mirzabey incubated in 4W2D medium, $B_1$ from 12 weeks old Yüreğir culture incubated in 6W2D medium, and $B_2$ shows Yüreğir regenerated callus ready to transfer root strength medium.

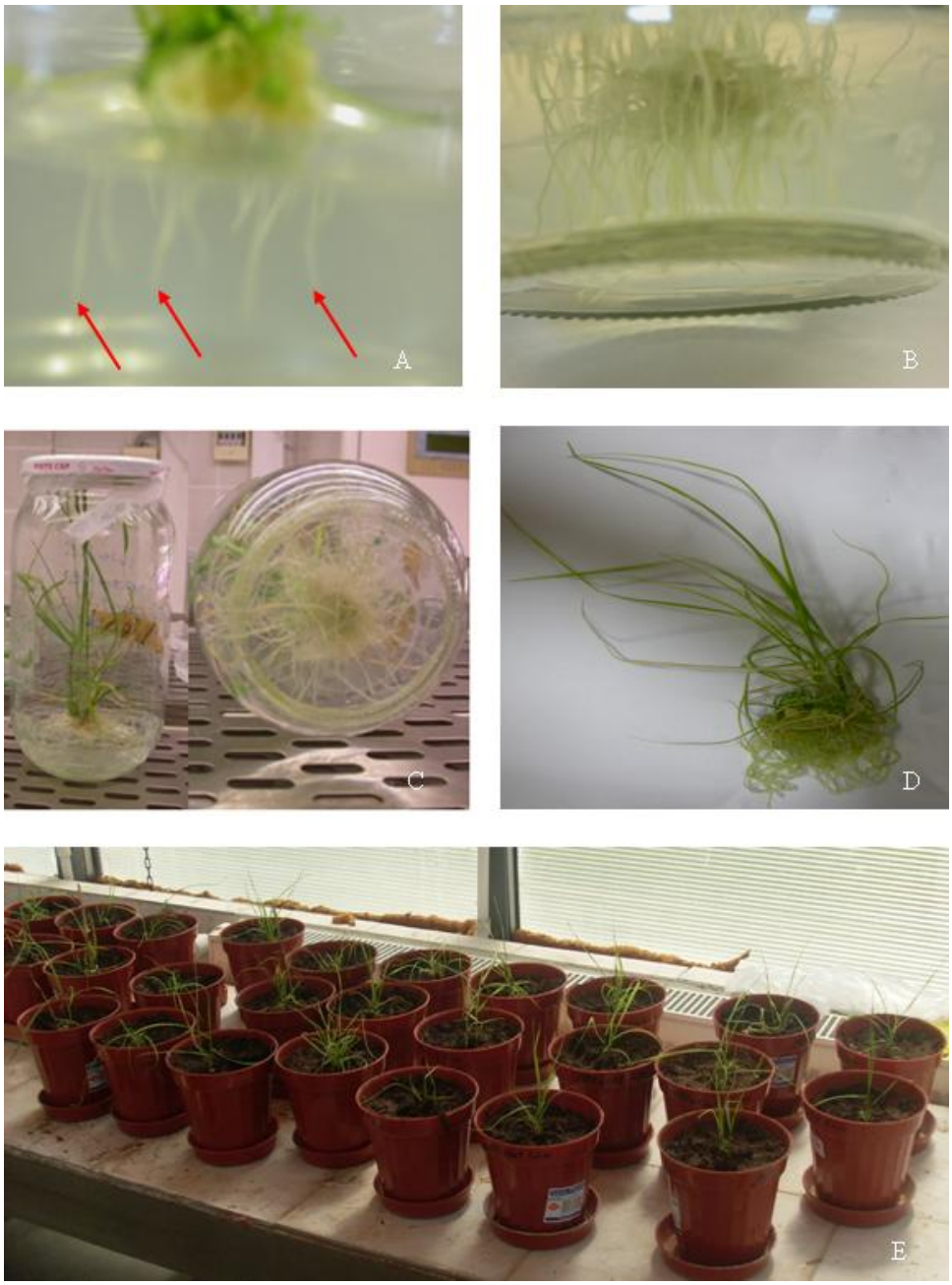


Figure 3. 15. Root strength medium and transfer to greenhouse. A shows 3 days after root strength medium incubation of Mirzabey. The red arrows shows root structures. B and C show 3 weeks incubated in root strength medium (nearly 15 weeks old plantlets of Mirzabey). D shows plantlet of Mirzabey ready to carry on greenhouse. E shows planted plantlets of Mirzabey 1 week after from soil transfer

Table 3. 13. Regeneration and culture efficiency rate for Mirzabey

| Factor[1] | | | Regeneration rate [2] | Culture efficiency |
|---|---|---|---|---|
| a | b | c | (%) | [3] (%) |
| 4w | 2 | 2,4-D | 62.31 a | 44.13 a |
| 4w | 4 | 2,4-D | 15.63 b | 5.13 c |
| 4w | 8 | 2,4-D | 0.00 | 0.00 |
| 4w | 2 | Picloram | 45.02 a | 27.83 ab |
| 4w | 4 | Picloram | 33.42 ab | 19.00 bc |
| 4w | 8 | Picloram | 16.34 b | 10.43 c |
| 6w | 2 | 2,4-D | 47.29 a | 39.25 ab |
| 6w | 4 | 2,4-D | 20.41 b | 9.13 c |
| 6w | 8 | 2,4-D | 0.00 | 0.00 |
| 6w | 2 | Picloram | 42.69 a | 32.63 ab |
| 6w | 4 | Picloram | 19.95 bc | 14.33 c |
| 6w | 8 | Picloram | 11.95c | 8.38 c |

Means denoted by different letters in a column are significantly different at $p< 0.05$ according to One-Way ANOVA test. [1]Factor a: dark incubation time; Factor b: hormone concentration mg/l; Factor c: hormone type. [2]No.Regenerated plantlets/No.Cultured callus onto regeneration medium X 100. [3]No. Rgenerated plantlets/ No.Mature embryo cultured onto induction medium X 100.

Figure 3.14 $A_1$ and $A_2$ show shoot formation and plant regeneration of Mirzabey in regeneration medium. Figure 3.15 shows root formation and greenhouse transfer of Mirzabey. The mature embryo based regeneration rate and culture efficiency of Mirzabey was demonstrated in Table 3.13. The regeneration frequency was the highest in 4W2D medium, 62.31 %. The rate was 47.29 % for 6W2D, 45.02 % for 4W2P, 42.69 % for 6W2P. The rate was determined between 15.63-20.41 % in including 4 mg/l 2,4-D media, 4W4D and 6W4D. For 8 mg/l 2,4-D, regeneration data was not recorded. The embryogenic capacity was 33.42 % for 4W4P, 19.95 %

for 6W4P, 16.34 % for 4W8P and 11.95 % for 6W8P. If culture efficiency was evaluated, the 4W2D medium having maximum regeneration capacity had significantly higher culture efficiency rate other media. For picloram, the maximum culture efficiency rate was in 6W2P (39.25 %) and the minimum in 6W8P medium (8.38 %).

Table 3. 14. Regeneration and culture efficiency rate for Yüreğir

| Factor[1] | | | Regeneration rate [2] (%) | Culture efficiency [3] (%) |
|---|---|---|---|---|
| a | b | c | | |
| 4w | 2 | 2,4-D | 0.00 | 0.00 |
| 4w | 4 | 2,4-D | 0.00 | 0.00 |
| 4w | 8 | 2,4-D | 0.00 | 0.00 |
| 4w | 2 | Picloram | 3.58 | 1.75 |
| 4w | 4 | Picloram | 1.98 | 1.4 |
| 4w | 8 | Picloram | 0.00 | 0.00 |
| 6w | 2 | 2,4-D | 5.00 | 1.75 |
| 6w | 4 | 2,4-D | 0.00 | 0.00 |
| 6w | 8 | 2,4-D | 0.00 | 0.00 |
| 6w | 2 | Picloram | 0.00 | 0.00 |
| 6w | 4 | Picloram | 0.00 | 0.00 |
| 6w | 8 | Picloram | 3.57 | 0.88 |

Means denoted by different letters in a column are significantly different at $p < 0.05$ according to One-Way ANOVA test. [1]Factor a: dark incubation time; Factor b: hormone concentration mg/l; Factor c: hormone type. [2]No.Regenerated plantlets/No.Cultured callus onto regeneration medium X 100. [3]No. Regenerated plantlets/ No.Mature embryo cultured onto induction medium X 100.

Figure 3.14 $B_1$ and $B_2$ show shoot formation and plant regeneration of Yüreğir in regeneration medium. The regeneration and culture efficiency of Yüreğir was given in Table 3.14. There was no regeneration record for 4W2D, 4W4D, 4W8D, 4W8P, 6W4D, 6W8D, 6W2P and 6W4P media. The maximum regeneration (5 %) was in 6W2D medium,. The rate was 3.58 % for 4W2P, 3.57 % 6W8P and 1.98 % for 4W4P. Culture efficiency was 1.75 % for 4W2P and 6W2D, 1.4 % for 4W4P and 0.88 % for 6W8P.

Table 3. 15. Two-way ANOVA analysis of plant regeneration

| **Independent variables** | **F** | **p** |
|---|---|---|
| Hormone type (H) | 1.32 | 0.253 |
| Concentration (C) | 36.37*** | 0.000 |
| Dark incubation (D) | 1.06 | 0.305 |
| Genotype (G) | 142.34*** | 0.000 |
| H * C | 4.33** | 0.015 |
| H * D | 0.60 | 0.441 |
| H * G | 0.61 | 0.437 |
| C * D | 0.20 | 0.818 |
| C * G | 34.33*** | 0.000 |
| D * G | 1.73 | 0.190 |

*p<0.05; **p<0.01; ***p<0.001

Bi and Wang (2008) reported regeneration rate between 2.17-32.40 % and culture efficiency was between 0.60- 27.70 %. Chen and colleagues (2006) evaluated the regeneration rate and culture efficiency of mature embryo derived calli incubated in 8 mg/l 2,4-D for 3 weeks. According to their results, regeneration rate was between 25-65.8 % and culture efficiency changed from 5.2 % to 21.7 %. In this study the minimum 8 mg/l 2,4-D incubation was 8 weeks and resulted with necrotic tissue structure. There was no regeneration record for this hormone concentration of 2,4-D. Chauhan and colleagues (2007) observed no regeneration in some hormone type and concentration both of bread and durum wheat. Yüreğir regeneration capacity in some conditions showed similar result with this study. According to Bi and colleagues (2007) study, the regeneration rate was maximum 50 % and the minimum 2,17 % from including 2 mg/l 2,4-D medium depending on different genotypes. In 2008, the regeneration rate was reported between 49.1-67.0 using 2 mg/l 2,4-D among the different genotypes (Yu et.al, 2008). In the same study culture efficiency was changed from 17.8 % to 36.8 %. Including 2 mg/l 2,4-D media gave the maximum regeneration rate and culture efficiency for 4 and 6 weeks dark incubated Mirzabey mature embryo based culture. However, there was no applicable regeneration rate and culture efficiency for Yüreğir in this study. Demirbaş (2004) reported a highly efficient regeneration system for Yüreğir using immature inflorescences as source of explants. According to Two-way ANOVA analysis (Table 3.15.), hormone type, concentration and genotype significantly affected plant regeneration from mature embryo based callus. The significantly effects of cultivar genotype, hormone type and concentration on plant regeneration were demonstrated Bi and Wang in 2008, Wei et.al, in 2003, Mendoza and Kaeppler in 2002. The regeneration results obtained in this study showed similarity with literature.

### 3.1.3.1. Average shoot number and root formation

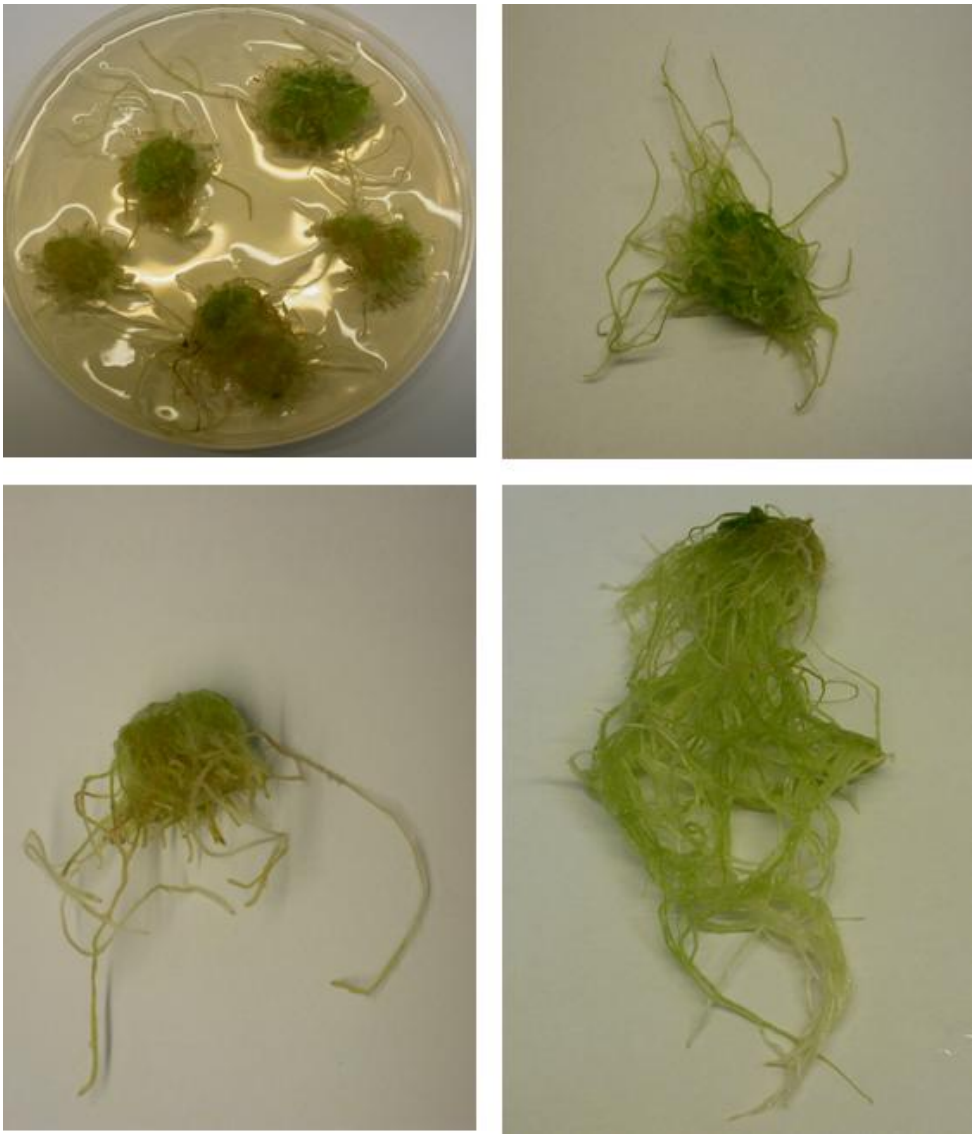

Figure 3. 16.Rooted and non-embryogenic calli of Yüreğir from regeneration medium

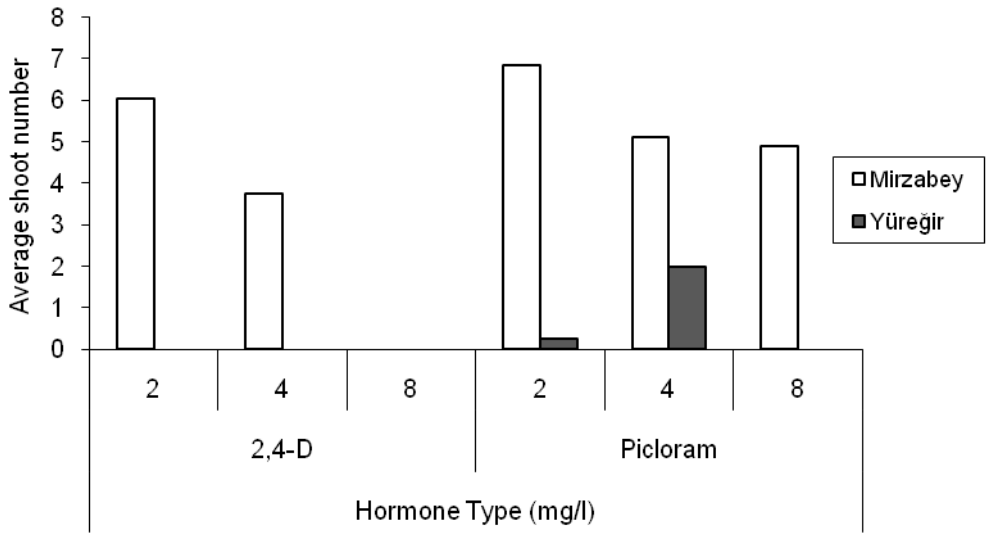


Figure 3. 17.Average shoot number of 4 weeks dark incubated calli

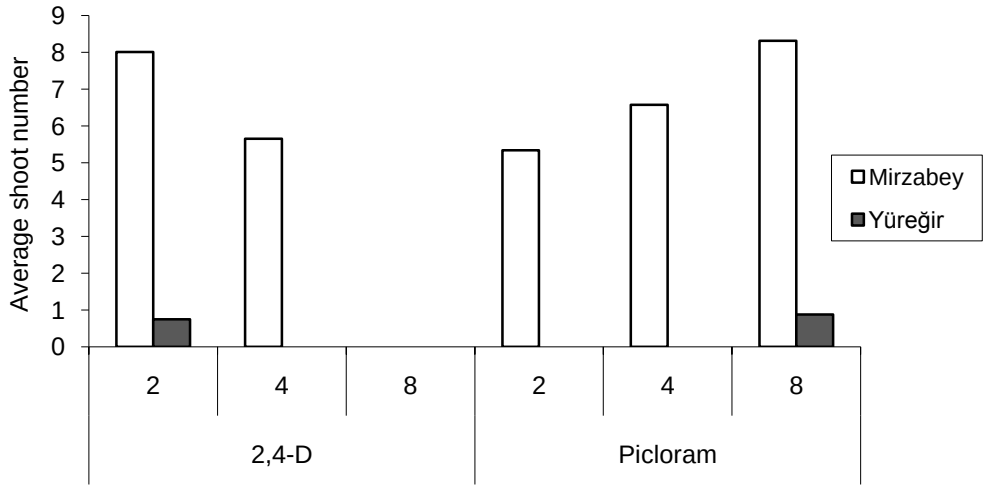


Figure 3. 18.Average shoot number of 6 weeks dark incubated calli

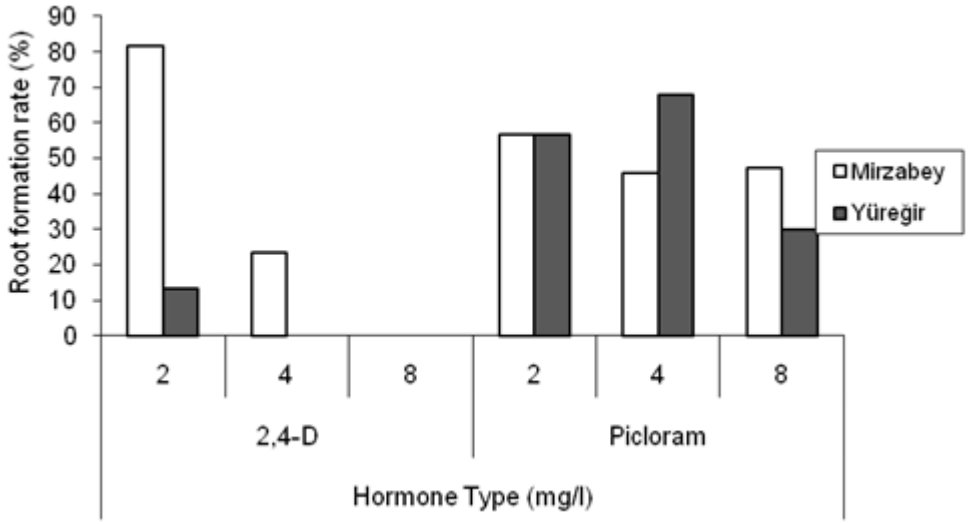


Figure 3. 19.Average root formation rate of 4 weeks dark incubated cali

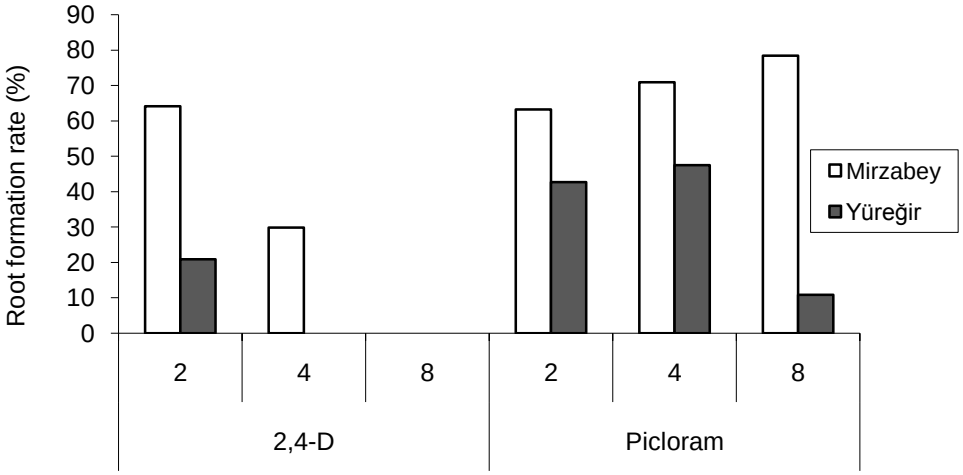


Figure 3. 20.Average root formation rate of 6 weeks dark incubated calli

Table 3. 16.Average shoot number per plantlets and root formation rate for Mirzabey

| Factor[1] | | | Average shoot number per plantlets[2] | Root formation rate [3] (%) |
|---|---|---|---|---|
| a | b | c | | |
| 4w | 2 | 2,4-D | 6.04 | 81.63 |
| 4w | 4 | 2,4-D | 3.75 | 23.38 |
| 4w | 8 | 2,4-D | 0.00 | 0.00 |
| 4w | 2 | Picloram | 6.85 | 56.50 |
| 4w | 4 | Picloram | 5.10 | 45.67 |
| 4w | 8 | Picloram | 4.90 | 47.43 |
| 6w | 2 | 2,4-D | 8.01 | 64.13 |
| 6w | 4 | 2,4-D | 5.65 | 29.88 |
| 6w | 8 | 2,4-D | 0.00 | 0.00 |
| 6w | 2 | Picloram | 5.34 | 63.25 |
| 6w | 4 | Picloram | 6.57 | 70.88 |
| 6w | 8 | Picloram | 8.31 | 78.38 |

[1]Factor a: dark incubation time; Factor b: hormone concentration mg/l; Factor c: hormone type. [2]No.Shoot on regenerated plantlets/No.Rgenerated plantlets. [3]No.Callus with root/ No.Mature embryo cultured onto induction medium X 100.

According to Figure 3.17 and 3.18 and Table 3.16, the maximum average shoot number per plantlets produced Mirzabey mature embryo based callus was determined in 6W8P medium, 8.31. The shoot formation rate was 6.04 in 4W2D medium having maximum regeneration rate. The minimum shoot formation rate was reported in 4W4D medium. There was no shoot and root formation for 8 mg/l 2,4-D medium because of its detrimental effect. The root formation rate for Mirzabey was the highest in 4W2D medium, Figure 3.19 and 3.20. Picloram including media gave the variable results between 45.67-78.38 % for root formation rate.

Table 3. 17. Average shoot number per plantlets and root formation rate for Yüreğir

| Factor[1] | | | Average shoot number per plantlets[2] | Root formation rate[3] |
|---|---|---|---|---|
| a | b | c | | |
| 4w | 2 | 2,4-D | 0.00 | 13.38 |
| 4w | 4 | 2,4-D | 0.00 | 0.00 |
| 4w | 8 | 2,4-D | 0.00 | 0.00 |
| 4w | 2 | Picloram | 0.25 | 56.75 |
| 4w | 4 | Picloram | 2.00 | 68.00 |
| 4w | 8 | Picloram | 0.00 | 30.00 |
| 6w | 2 | 2,4-D | 0.75 | 20.88 |
| 6w | 4 | 2,4-D | 0.00 | 0.00 |
| 6w | 8 | 2,4-D | 0.00 | 0.00 |
| 6w | 2 | Picloram | 0.00 | 42.71 |
| 6w | 4 | Picloram | 0.00 | 47.50 |
| 6w | 8 | Picloram | 0.88 | 10.88 |

[1]Factor a: dark incubation time; Factor b: hormone concentration mg/l; Factor c: hormone type. [2]No.Shoot on regenerated plantlets/No.Regenerated plantlets. [3]No.Callus with root/ No.Mature embryo cultured onto induction medium X 100.

According to Figure 3.17 and 3.18 and Table 3.17., the maximum shoot number (2.00) and root formation rate (68.00 %) were observed in 4W4P medium. There was no shoot and root formation including 4 and 8 mg/l 2,4-D media. The minimum root formation rate was in 4W2D medium, Figure 3.19 and 3.20.

Filippov and colleagues (2006) reported average shoot number changing between 3.2 and 8.8 in high concentration of 2,4-D. In this study, 4 mg/l 2,4-D media average shoot number was varied between 3.75 and 5.34 and no shoot formation in 8 mg/l

2,4-D media for Mirzabey. They only incubated calli 3 weeks in high concentration of 2,4-D and transferred to regeneration medium. Mendoza and Kaeppler (2002) found average shoot formation for 2 mg/l 2,4-D between 0.1-0.5, no formation for 4 mg/l similar result for Yüreğir, for 2 mg/l picloram between 0.8-1.2 and for 4 mg/l picloram from 0.1 to 1.1. When Mirzabey average shoot number results given in Table 3.16 were evaluated, the average shoot number was higher than Mendoza and Kaeppler. Because, the silver nitrate was used in this study to promote shoot induction as reported by Yu and colleagues (2008).

### 3.1.3.2. Determination of vernalization period for Mirzabey-2000

The winter wheats need a vernalization period to gain capability of flowering. 1 week, 2 weeks and 3 weeks vernalized wheat did not complete flowering induction, Figure 3.21. The spike formation was not observed. Hovewer, 4 weeks and 5 weeks vernalized wheat flowered and harvested. After these observations 30 days of + 4ºC was determined the best vernalization period for Mirzabey cultivar. Yüreğir does not need to vernalization, because it is a spring wheat cultivar.

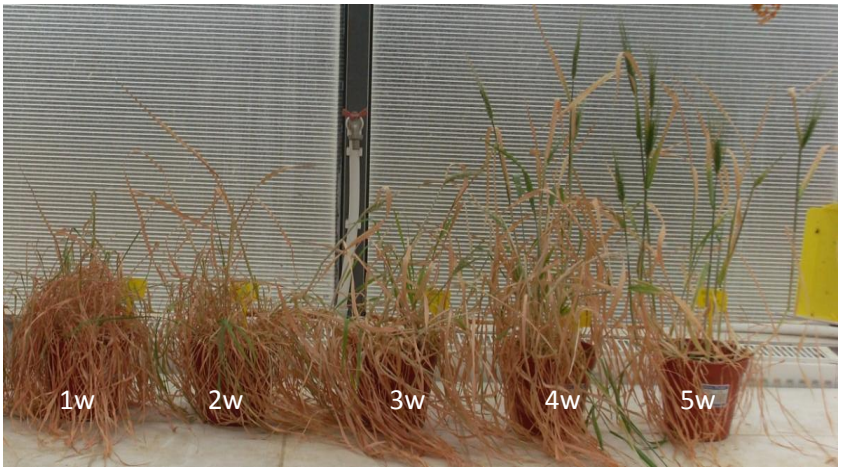


Figure 3. 21. Mirzabey plants after vernalization period applications. Vernalization time: 1w: 1 week; 2w: 2weeks; 3w: 3weeks; 4w: 4 weeks; 5w: 5weeks

### 3.1.3.3. Seed characteristics

After transferred to greenhouse, Yüreğir plantlets were maturated in 2 months and Mirzabey plantlets vernalized 1 month were grown in 3 months (Fig. 3.22). Yüreğir and Mirzabey plantlets gave the healthy and normal spikes and seeds. However, the normal and abnormal spikes and seeds were also harvested from both of cultivars (Fig 3.23 and 3.24).

Figure 3. 22. 3 months old Mirzabey


Figure 3. 23. Seed appearances for two cultivars. $A_1$ shows normal seeds and $A_2$ shows abnormal seeds from Mirzabey. $B_1$ shows normal seeds and $B_2$ shows abnormal seeds from Yüreğir.


Figure 3. 24. Spike appearances for two cultivars. $A_1$ shows normal spikes and $A_2$ shows abnormal spikes from Mirzabey. $B_1$ shows normal spikes and $B_2$ shows abnormal spikes from Yüreğir.

### 3.2.Transformation studies

In the transformation study, firstly, plasmid was isolated and, then, confirmed by single and double cutting and, lastly, bombarded mature embryo derived calli via old and modified loading units.

### 3.2.1. Single and double digestion of the plasmid

After plasmid isolation plasmid was restricted *SmaI* and *SacI* restriction enzymes. *pAHC25* length is 9706 bp. Plasmid is cut only one restriction site for *SmaI* (at 2022 bp) and *SacI* (at 3910 bp). The linear form of the plasmid can be constituted using one of them. If the both of enzymes are used together, the plasmid is cut double digested and *gus* gene (1888 bp) is removed. The Figure 3.25 shows *pAHC25* restriction results. According to gel electrophoresis result, the plasmid was isolated correctly and ready to use for bombardment.

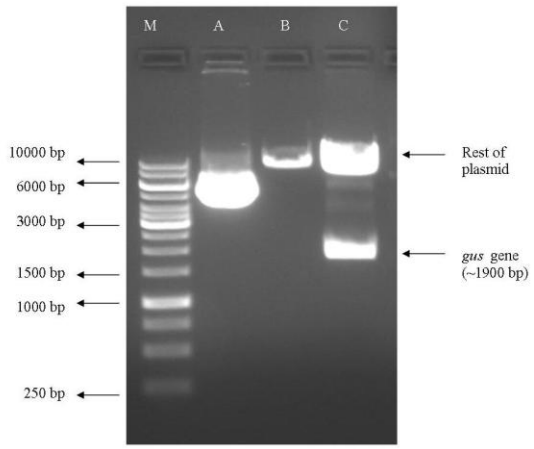


Figure 3. 25. Agarose gel electrophoresis of restricted pAHC25 with *SmaI* and *SacI*. M: Marker gene (Range: 250-10000 bp), A: Control (No restriction or circular DNA), B: Restriction with *SmaI* (Linear form of the plasmid), C: Restriction with *SmaI* and *SacI* (*gus* gene and rest of the plasmid)

### 3.2.2. The old loading unit

The plasmid *pAHC25* coated gold particles was bombarded 30, 35, 40 and 45 bar pressure and 8 and 10 cm distance using old loading unit. The no DNA coated gold particles were used as control. After histochemical GUS assay, the transient gus expression was observed on some calli for all conditions and no expression for control explants.

The calli having blue spots and total blue spots were counted. The Table 3.18 shows transformation results for the old loading unit. The maximum number of calli (12) expressing *gus* gene was 30 bar pressure and 10 cm distance. Blue spot number was 34 for this condition. The maximum blue spot number (70) was detected for 30 bar pressure and 8 cm distance. If transformation efficiency was evaluated, the highest rate was also for 30 bar pressure and 8 cm distance. The callus number expressing gus gene was between 2-8, the number of blue spots was between 7-22 and transformation efficiency was between 0.14-0.76 for other pressure and distance combinations.

According to results, the best transformation efficiency was observed for 30 bar pressure and 8 cm distance using the old loading unit. Previous studies performed other colleagues in our laboratory gave the best results for pressure between 30-35 bar and 8 cm for distance. Thus, 30 and 35 bar for and 8 cm were used to test modified loading unit.

Table 3. 18. Transformation results for old loading system

| Factors | | | | | | |
|---|---|---|---|---|---|---|
| **Pressure(bar)** | **Distance(cm)** | **a** | **b** | **c** | **d** | **e** |
| 30 | 8 | 11/45 | 70 | 0.24 | 6.36 | 1.53 |
| | 10 | 12/45 | 34 | 0.27 | 2.83 | 0.76 |
| 35 | 8 | 8/45 | 22 | 0.18 | 2.75 | 0.50 |
| | 10 | 2/45 | 7 | 0.04 | 3.50 | 0.14 |
| 40 | 8 | 6/45 | 9 | 0.13 | 1.50 | 0.20 |
| | 10 | 7/45 | 22 | 0.16 | 3.14 | 0.50 |
| 45 | 8 | 8/45 | 13 | 0.18 | 1.63 | 0.29 |
| | 10 | 7/45 | 13 | 0.16 | 1.86 | 0.30 |

Factors: a:No.Callus expressed GUS, b:No.Blue spots, c:No.Callus expressed GUS / No.Bombarded callus, e:No.Blue spots / No.Callus expressed GUS, e:Transformation efficiency = c X d

### 3.2.3. The modified loading unit:

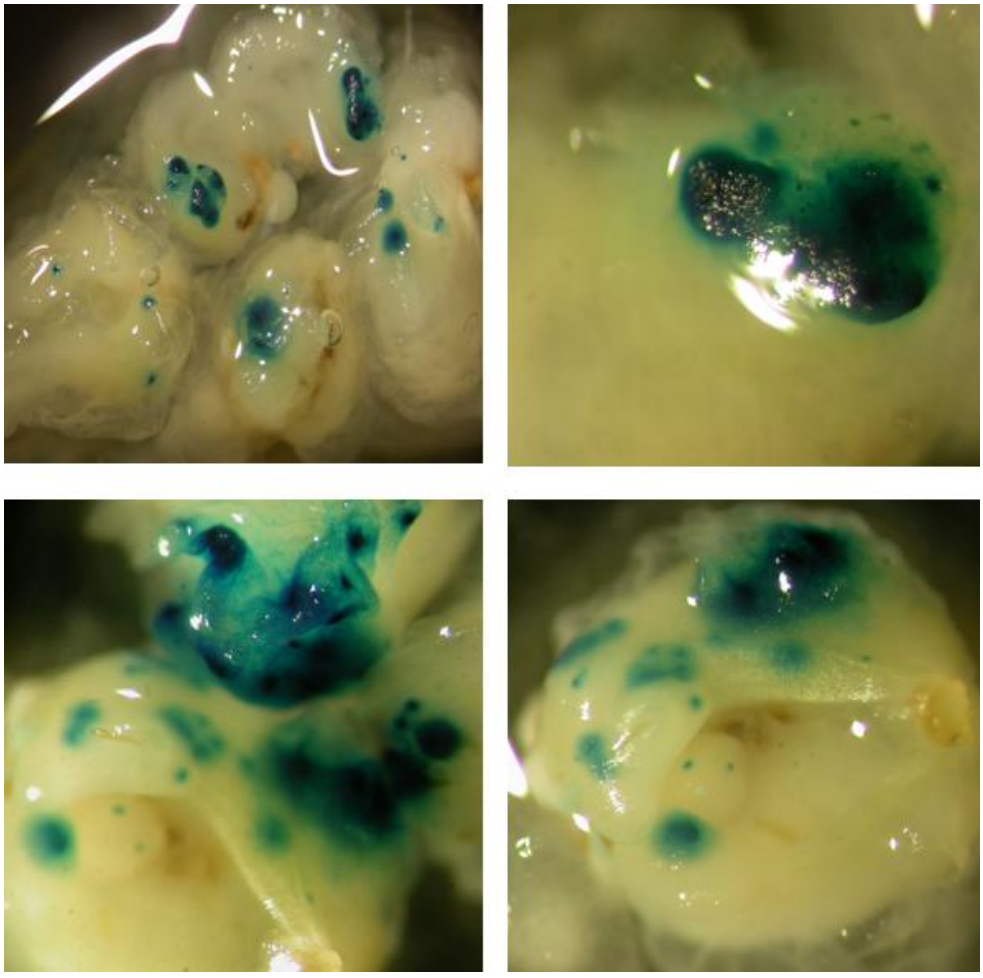

Figure 3. 26. Transient gus exprssion results using the modified loading system

Table 3. 19. Transformation results for the modified loading unit

| Factors | | | | | | |
|---|---|---|---|---|---|---|
| **Pressure(bar)** | **Distance(cm)** | **a** | **b** | **c** | **d** | **e** |
| 30 | 8 | 12/30 | 75 | 0.40 | 6.25 | 2.50 |
| 35 | 8 | 11/30 | 66 | 0.37 | 6.00 | 2.22 |

Factors: a:No.Callus expressed GUS, b:No.Blue spots, c:No.Callus expressed GUS / No.Bombarded callus, e:No.Blue spots / No.Callus expressed GUS, e:Transformation efficiency = c X d

While 12 calli expressed *gus* gene for 30 bar pressure and 8 cm distance, 11 calli expressed for 35 bar. The blue spot number was 75 for 30 bar and 66 for 35 bar. If transformation efficiency was evaluated, transformation efficiency was 2.50 for 30 bar and 2.22 for 35 bar.

When we compare the old and the modified loading units, the transformation efficiency increased from 1.53 to 2.50, nearly 0.65 fold increases, for 30 bar and 8 cm. For 35 bar and 8 cm, transformation efficiency increased from 0.50 to 2.22, nearly 5.6 fold increases. Figure 3.26 shows transient GUS expression using modified unit. According to these results, the modified loading is more effective than the old loading unit.

Oard and colleagues (1990) found 7 cm distance was optimal for maize suspension culture and rice callus culture. They found 0.25 or 2.5 average blue spots per embryo for maize. This rate was between 0.16 and 2.5 for this study. Particle size, gas pressure and distance were studied by Dobrzahska and colleagues in 1997. They found average number of blue spots per callus expressing gus gene between 0.1 and 19.8. The maximum transient expression was for 76 bar and 9 cm. However, they

used Biorad gene transfer system reported for many successful transformation and they resuspended DNA coated tungsten in small amount of volume (60µl). 100 µl was used resuspension volume for DNA coated gold particles in this study. Also, they used 10 µl of loading solution for per shot and we used 8 µl of loading solution. These mean that the bombarded DNA coated gold particles were lower than Dobrzahska and colleagues. Öktem and colleagues (1999) achieved more than 80 % transient expression of gus gene on the bombarded wheat mature embryos. This rate was between 4-27 % for the old loading unit and 37-40 % for the modified loading unit. This rate increased from 18 % to 37 % for 35 bar and 8 cm using the modified loading system. They used Genebooster gene transfer system and bombarded two times with 5 µl of loading solution. We bombarded one shot per plate using 8 µl of loading solution. In case the bombarded gold particles amount was lower than Öktem and colleagues. Folling and Olesen (2001) reported helium gas pressure, distance and amount of DNA coated microprojectile affected transient gus expression. The pressure and distance also affected transformation efficiency in this study. There was different transformation efficiency for the same pressure condition, such as at 30 bar pressure for 8 cm was 1.53 and for 10 cm was 0.76 using the old loading system. Also, there was different transformation frequency at the same distance and different pressure, such as at the 8 cm 1.53 was for 30 bar and 0.50 for 35 bar using old loading unit. However, this rate was increased using the modified loading system. As a result, this modified developed loading unit can be used for transformation studies to introduce new desirable traits to wheat genome.

# CHAPTER 4

# CONCLUSION

In this study, the regeneration paramaters of mature embryo based cultures of pasta wheat cultivar Mirzabey 2000 (*Triticum durum*) and bread wheat cultivar Yüreğir 89 (*Triticum aestivum*) were optimized. The effects of auxin hormone types and concentrations and dark incubation periods were investigated.

- The average callus fresh weight after callus induction medium incubation was between 44.00-86.38 mg for 6 weeks old and dark incubated Mirzabey calli. Primary callus induction rate was determined 93.25 % in 6W2P medium for 6 weeks old and dark incubated Mirzabey calli.

- The average callus fresh weight of 6 weeks old and dark incubated Yüreğir calli was between 25.25-44.00 mg. The maximum primary callus induction was observed in 6W2D medium for this condition.

- The average callus fresh weight after 6 weeks dark incubation was the minimum in 6W8D medium for both of cultivars.

- The average callus fresh weight after callus induction medium incubation was between 33.83-43.38 mg for 4 weeks old and dark incubated Mirzabey calli. Primary callus induction rate was determined 91.00 % in 4W4P medium for 4 weeks old and dark incubated callus.

- The average callus fresh weight of 4 weeks old and dark incubated Yüreğir calli was between 12.80- 22.50 mg. Primary callus induction rate was 86.75 % in 4W8P medium.

- The average callus fresh weight of 4 weeks old and dark incubated Mirzabey cultivar was not affected hormone type and concentration and their together effect. However, 6 weeks old and dark incubated average callus fresh weight was significantly affected hormone type ($p<0.05$), concentration ($p<0.001$) and their together effect ($p<0.01$).

- Hormone type and concentration significantly affected both of 4 and 6 weeks old and dark incubated Yüreğir average callus fresh weight.

- While hormone type and concentration did not significantly affect primary callus induction of 4 and 6 weeks old and dark incubated Mirzabey culture, they significantly affected Yüreğir 4 and 6 weeks old and dark incubated calli. Hormone type, concentration and genotype significantly affect primary callus induction.

- The average callus fresh weight after embryogenic callus induction medium was the minimum in 6W8D medium and the maximum in 6W2D medium for Mirzabey calli 10 weeks old 6 weeks incubated at dark before. The embryogenic callus induction rate also gave the same results, 94.88 % for 6W2D and 5.13 % for 6W8D. Detrimental effect of high concentration of 2,4-D was observed in 6W8D and 6W4D media.

- The 10 weeks old 6 weeks dark incubated before Yüreğir calli gave the similar results with Mirzabey for the average callus fresh weight and embryogenic callus induction rate. It was recorded that 26.88 % from 6W2D the highest rate. There was no embryogenic callus formation for 6W4D and 6W8D media. The necrotic tissues (100 %) were observed in these media.

- For 8 weeks old and 4 weeks dark incubated Mirzabey calli gave the maximum results in 4W2D medium. Also, the lowest results came from including 8 mg/l 2,4-D medium. There was also necrosis in this medium.

- For Yüreğir calli 8 weeks old and 4 weeks dark incubated, the maximum average callus fresh weight was in 4W4P and embryogenic callus induction rate was in 4W2D medium. Like 10 weeks old calli, there was no embryogenic capacity for 4W4D and 4W8D media. Also, browning structure called necrosis was observed.

- The average callus fresh weight after embryogenic callus induction media and embryogenic capacity of calli were significantly affected hormone type, concentration and genotype.

- There was no record of regeneration of Mirzabey calli in 6W8D and 4W8D media. The maximum regeneration rate (62.31 %) was observed in 4W2D medium and also, culture efficiency (44.13 %) was the highest for this medium. The rate was 47.29 % for 6W2D, 45.02 % for 4W2P and 42.69 % for 6W2P media.

- There was no highly regeneration for Yüreğir mature embryo based culture in this study. The maximum rate was 5 % in 6W2D medium.

- According to analyses of variance, regeneration rate was significantly affected from hormone concentration, genotype, hormone type and concentration and genotype and concentration.

- The average shoot number of Mirzabey culture was the highest for 6W8P medium, 8.31. The number was 8.01 for 6W2D, 6.85 for 4W2P, 6.57 for 6W4P and 6.04 for 4W2D media. Root formation was not observed including

8 mg/l 2,4-D because of its detrimental effect. 81.63 % root formation was recorded from 4W2D medium.

- For Yüreğir, the shoot formation rate was the maximum 2.0 in 4W4P medium. There was no root formation in including 4 and 8 mg/l 2,4-D media. The maximum root formation was also observed in 4W4P medium.

- After transferred to greenhouse, Yüreğir plantlets were maturated in 2 months and Mirzabey plantlets vernalized 1 month were grown in 3 months. Yüreğir plantlets gave the healty and normal spikes and seeds. However, the normal and abnormal spikes and seeds were harvested from Mirzabey plants.

- As a conclusion, the usage of including 2 mg/l 2,4-D medium was important for primary callus induction, embryogenic callus induction and regeneration in mature embryo based wheat tissue culture studies. The use of high concentration of 2,4-D caused browning of tissues and death of calli and decreased the regeneration potential. The picloram can be used as an alternative hormone type instead of 2,4-D. The further optimization studies must be performed to increase effect of picloram.

In transformation study, the optimum conditions of velocity and distance were investigated using old and modified loading units of particle bombardment device to transfer foreign gene to mature embryo based wheat calli.

- The old loading unit was tested at bombarding pressure of 30, 35, 40 and 45 bar and 8 and 10 cm distance. The maximum transformation efficiency was recorded at 30 bar pressure and 8 cm distance.

- The modified system was tested at bombarding pressure of 30 and 35 bar and 8 cm distance. The transformation efficiency based transient gene expression increased 0.65 fold for 30 bar and 5.6 fold for 35 bar.

- As a result, the modified loading system can be used as a part of bombardment unit to produce genetically modified organisms.

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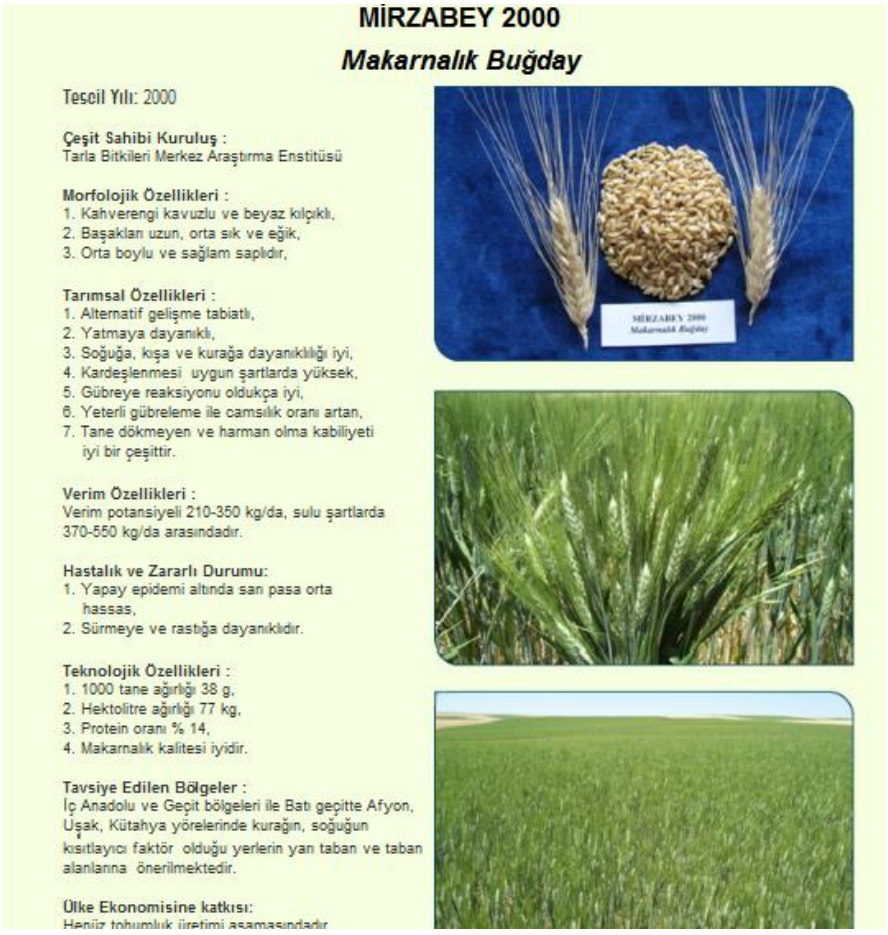
# APPENDIX A

## INFORMATION ON MİRZABEY-2000 AND YÜREĞİR-89



**MİRZABEY 2000**

***Makarnalık Buğday***

Tescil Yılı: 2000

**Çeşit Sahibi Kuruluş :**
Tarla Bitkileri Merkez Araştırma Enstitüsü

**Morfolojik Özellikleri :**
1. Kahverengi kavuzlu ve beyaz kılçıklı,
2. Başakları uzun, orta sık ve eğik,
3. Orta boylu ve sağlam saplıdır,

**Tarımsal Özellikleri :**
1. Alternatif gelişme tabiatlı,
2. Yatmaya dayanıklı,
3. Soğuğa, kışa ve kurağa dayanıklılığı iyi,
4. Kardeşlenmesi uygun şartlarda yüksek,
5. Gübreye reaksiyonu oldukça iyi,
6. Yeterli gübreleme ile camsılık oranı artan,
7. Tane dökmeyen ve harman olma kabiliyeti iyi bir çeşittir.

**Verim Özellikleri :**
Verim potansiyeli 210-350 kg/da, sulu şartlarda 370-550 kg/da arasındadır.

**Hastalık ve Zararlı Durumu:**
1. Yapay epidemi altında sarı pasa orta hassas,
2. Sürmeye ve rastığa dayanıklıdır.

**Teknolojik Özellikleri :**
1. 1000 tane ağırlığı 38 g,
2. Hektolitre ağırlığı 77 kg,
3. Protein oranı % 14,
4. Makarnalık kalitesi iyidir.

**Tavsiye Edilen Bölgeler :**
İç Anadolu ve Geçit bölgeleri ile Batı geçitte Afyon, Uşak, Kütahya yörelerinde kurağın, soğuğun kısıtlayıcı faktör olduğu yerlerin yarı taban ve taban alanlarına önerilmektedir.

**Ülke Ekonomisine katkısı:**
Henüz tohumluk üretimi aşamasındadır

Figure A.1. Mirzabey cultivar, http://www.tarlabitkileri.gov.tr/cesitlerimiz/bugday/makarnalik, 04.09.2010

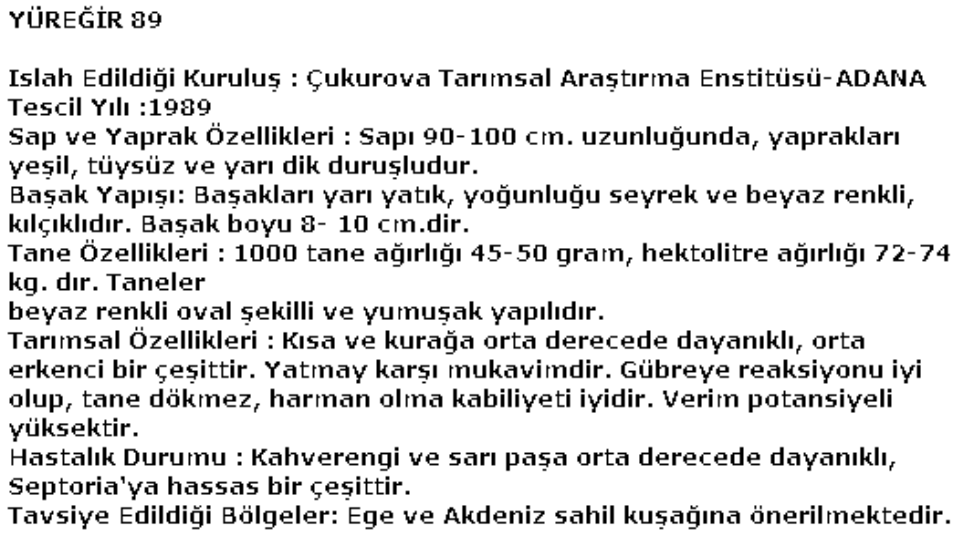



Figure A.2. Yüreğir cultivar,

http://site.mynet.com/mustafababuroglu/mustafababuroglu/id3.htm, 04.09.2010

## APPENDIX B

## AGRONOMICALLY IMPORTANT GENES TRANSFERRED INTO WHEAT

Table B.1. Agronomically important genes transferred into wheat (Adopted from Sarawat et al., 2003)

| **Target tissue** | **Source of the gene** | **Gene** | **Selectable Marker** | **Phenotype** | **References** |
|---|---|---|---|---|---|
| IE | Barley yellow mosaic virus | Coat protein (cp) | *Bar* | No data on phenotype | Karunarante *et al.*, 1996 |
| IE | *T. aestivum* L. | High molecular weight glutenin subunit (1Ax1) | *Bar* | Accumulation of glutenin subunit 1Ax1 | Altpeter *et al.*, 1996 |
| IE | *T. aestivum* L. | High molecular weight glutenin hybrid subunits (Dy10:Dx5) | *Bar* | Accumulation of hybrid glutenin subunit | Blechl and Anderson, 1996 |
| EC | *Bacillus amyloliguefaciens* | Barnase | *Bar* | Nuclear male sterility | Sivamani *et al.*, 2000 |
| IE | *T. aestivum* L. | High molecular weight glutenin subunits Dx5, 1Ax1 | *Bar* | Increased dough elasticity | Barro *et al.*, 1997 |

Table B.1 Continued

| | | | | | |
|---|---|---|---|---|---|
| IE | *Vitis vinifera* | Stilbene synthase (Vst1) | *Pat* | No data on resitance to fungus diseases | Leckband and Lörz, 1998 |
| IE | *T. aestivum* L. | High molecular weight glutenin hybrid subunits (Dy10:Dx5) | *Bar* | Accumulation of hybrid glutenin subunit | Blechl *et al.*, 1998 |
| IE | *Oryza sativa* | Rice chitinase | *Bar* | No data on phenotype | Chen *et al.*, 1998 |
| EC | *Hordeum vulgare* L. | Class II chitinase (*chiII*) | *Bar* | Resistance to fungus (*E. Graminis* ) | Bliffeld *et al.*, 1999 |
| IE | *O. sativa* | Thaumatinlike protein (*tlp*), chitinase (*chi11*) | *bar, hpt* | Resistance to fungus (*F. graminearum*) | Chen *et al.*, 1999 |
| IE | *Zea mays* | Transposase (Ac) | *Bar* | Synthesis of an active transposase protein in transgenic Ac line | Stöger *et al.*, 2000 |
| IE | *T. aestivum* L. | High molecular weight glutenin subunit (1Dx5) | | *Increased dough strength* | Rooke *et al.*, 1999 |

Table B.1 Continued

| | | | | | |
|---|---|---|---|---|---|
| IE | *T. aestivum* L. | High molecular weight glutenin subunits (1Axx1, 1Dx5) | *Bar* | Increased dough strength and stability | He *et al.*, 1999 |
| IE | *H. vulgare* L. | Trypsin inhibitor (CMe) | *Bar* | Resistance to angoumois grain moth (*S. cerealella* ) | Altpeter *et al.*, 1999 |
| IE | *Galanthusnivalis agglutinin* (GNA) | Agglutinin (*gna*) | *Bar* | Decreased fecundity of aphids (*Sitobin avanae*) | Stöger *et al.*, 1999 |
| IE | *H. vulgare* L. | Chimeric stilbene synthase gene (*sts*) | *Bar* | Production of phytoalexin resveratrol, no data on resistance to fungus diseases | Fettig and Hess, 1999 |
| IE | Wheat streak mosaic virus | Replicase gene (*NIb*) | *Bar* | Resistance to wheat steak mosaic virus (WSMV) | Sivamani *et al.*, 2000 |
| IE | *H. vulgare* L. | HVA1 | *Bar* | Improved biomass productivity and water use efficiency | Sivamani *et al.*, 2000 |

Table B.1 Continued

| | | | | | |
|---|---|---|---|---|---|
| IE | Monoclonal antibody | T84.66 Single chain Fv antibody (ScFvT84.66) | *bar, hpt* | Production of functional recombinant antibody in the leaves | Stöger *et al.*, 2000 |
| EC | *U. maydis* infecting virus | Antifungal protein (KP4) | *Bar* | Resistance against stinking smut | Clausen *et al.*, 2000 |
| IE | *A. niger* | Phytaseencoding gene (*PhyA*) | *bar* | Accumulation of phytage in transgenic seeds | Brinch-Pedersen *et al.*, 2000 |
| IE | *H. vulgare* L. | Ribosomeinactivating protein (*RIP*) | *Bar* | Moderate resistance to fungal pathogen *E. Graminis* | Bieri *et al.*, 2000 |
| IE | *Tritordeum*, tomato, oat | S-adenosyl methionine decarboxylase gene (SAMDC), arginine decarboxylase gene (ADC) | *Bar* | No data on phenotype | Bieri *et al.*, 2000 |
| IE. | *T. aestivum* L | High molecular weight glutenin subunits (1Ax1, 1Dx5) | | Flours with lower mixing time, peak resistance and sedimentation volumes | Alvarez *et al.*, 2001 |
| IE | Bacterial ribonulease III, wheat streak mosaic virus | Bacterial ribonulease III (rnc70), coat protein (cp) | *Bar* | No data on phenotype | Zhang *et al.*, 2001 |

Table B.1 Continued

| | | | | | |
|---|---|---|---|---|---|
| IE | *A. giganteus, H. vulgare* | Antifungal protein afp from *A. giganteus* , a barley class II chitinase and rip I | *Bar* | Inhanced fungal resistance | Oldach *et al.*, 2001 |
| IE. | *T. aestivum* L | FKBP73 WFKBP77 | *Bar* | Alteration in grain weight and composition in transgenic seeds | Kurek *et al.*, 2002 |
| IE | *T. aestivum* L. | High molecular weight glutenin subunits (1Ax1, 1Dx5) | *bar* | No data on phenotype | Barro *et al.*, 2002 |
| IE. | (soft wheat) *T. aestivum* L | Protein puroindoline (PinB-D1a) | *Bar* | Increased friabilin levels and decreased kernel hardness | Beecher *et al.*, 2002 |

Table B.1 Continued

| | | | | | |
|---|---|---|---|---|---|
| EC | *F. sporotrichioides* | *Fusarium sporotrichioides* gene (FsTRI101) | *Bar* | Increased resistance to FHB (*F. graminearum*) | Okubara *et al.*, 2002 |
| IPS | *Vigna aconitifolia* | D1-pyrroline-5-carboxylate synthetase (P5CS) | *nptI* | Increased tolerance to salt | Sawahel *et al.*, 2002 |
| IPS | Wheat streak mosaic virus | Coat protein gene (CP) | *Bar* | Various degree of resistance to wheat streak mosaic virus | Sivamani *et al.*, 2002 |

Abbreviations: IE, immature embryos; EC, embryogenic callus; IPS, indirect pollen system (in this system Agrobacterium suspension is pipetted on spikelets just before anthesis); bar , phosphinothricin acetyl transferase; nptII , neomycin phosphotransferaseII; hpt , hygromycin phosphotransferase; Dy10, a high molecular weight glutenin subunit (HMW-GS) gene sequence.

# APPENDIX C

## COMPOSITION OF PLANT TISSUE CULTURE MEDIA

Table. C1. Composition of plant tissue culture media.

| **COMPONENT** | mg/l |
|---|---|
| Ammonium nitrate | 650.0 |
| Boric acid | 6.2 |
| Calciumchloride anhydrous | 332.2 |
| Cobalt chloride. 6 $H_2O$ | 0.025 |
| Cupric sulphate.5 $H_2O$ | 0.025 |
| $Na_2EDTA$ | 37.26 |
| Ferrous sulphate. 7 $H_2O$ | 27.8 |
| Magnesium sulphate. $H_2O$ | 16.9 |
| Molybdic acid (sodium salt). $H_2O$ | 0.25 |
| Potassium iodide | 0.83 |
| Potassium nitrate | 1900.0 |
| Potassiumphosphate monobasic | 170.0 |
| Zinc sulphate. 7 $H_2O$ | 8.6 |
| **Organics** | |
| Glycine (free base) | 2.0 |
| Myo-inositol | 100.0 |
| Nicotinic acid (free acid) | 0.5 |
| Pyrodoxine. HCl | 0.5 |
| Thiamine. HCl | 0.1 |

## APPENDIX D

## HISTOCHEMICAL GUS ASSAY SOLUTIONS

Table D.1. GUS Substrate solution

| | |
|---|---|
| $NaPO_4$ buffer, pH = 7.0 | 0.1 M |
| EDTA, pH = 7.0 | 10 mM |
| Potassium ferricyanide, pH = 7.0 | 0.5 mM |
| X-Gluc | 1.0 mM |
| Triton X-100 | 0.1 % |

Table D.2. GUS Fixative solution

| | |
|---|---|
| Formaldehyde | 10 % (v/v) |
| Ethanol | 20 % (v/v) |
| Acetic acid | 5 % (v/v) |

# APPENDIX E

## BACTERIAL MEDIUM LURIA BROTH

Table E.1. Luria Broth medium

| | |
|---|---|
| Tryptone | 10 g |
| NaCl | 10 g |
| Yeast extract | 5 g |
| pH: 7.0 | |