OPTIMIZATION OF TISSUE CULTURE, REGENERATION AND *AGROBACTERIUM* MEDIATED TRANSFORMATION PARAMATERS IN WINTER WHEAT CULTIVARS (Kızıltan-91 and Bezostaja-01)

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ABSTRACT

OPTIMIZATION OF TISSUE CULTURE, REGENERATION AND *AGROBACTERIUM* MEDIATED TRANSFORMATION PARAMETERS IN WINTER WHEAT CULTIVAR (*Kızıltan-91 and Bezostaja-01*)

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The objective of this study was to optimize tissue culture and regeneration parameters of immature inflorescence culture of *Triticum aestivum* cv. Bezostaja-01 and *Triticum durum* cv. Kızıltan-91. The effects of callus age and vernalisation time of explants on regeneration success were evaluated. For determination of optimum vernalisation time of immature inflorescence, plants subjected to 4 ° C for 1, 2, 3, 4, and 5 weeks, respectively. Tillers containing immature inflorescences were collected at the same time. Percentage of inflorescence formed tillers over total explants were reached the highest value, 79 %, at 4 weeks cold treated Kızıltan cultivar and, 73 %, at 5 weeks cold treated Bezostaja cultivar. Isolated immature inflorescences were put onto 2mg /L 2,4-dichlorophenoxyacetic acid and picloram containing callus induction medium for Kızıltan and Bezostaja cultures, respectively. Callus induction rate were found to be 100 % for Kızıltan

and Bezostaja. These explants were taken to regeneration after 6, 9, 12 and 15 weeks of dark incubation period. The regeneration capacities of calli were determined as shooting percentage and data were collected after 4, 8, 12, and 15 week regeneration period. The highest shooting percentage of 69 %, were obtained from 6 weeks old calli produced from 4 weeks vernalised explants in Kızıltan cultures at the end of 15 weeks regeneration period. However, shooting percentage was 57.2 % for 9 weeks old calli while it decreases to 37.6 % in 12 weeks old calli and 44.2 % in 15 weeks old calli at the end of 15 weeks regeneration period. This showed that prolonged dark incubation period decreased regeneration capacity of the callus. However, there was no significant difference in regeneration capacities of calli produced from Bezostaja immature inflorescence and the highest shooting percentage was obtained from 9 weeks old calli produced from 5 weeks vernalised explants, 27.4 %.

Besides regeneration studies, optimization of transformation parameters for winter wheat cultivars Kızıltan and Bezostaja by using *Agrobacterium tumefaciens* AGLI containing binary vector pALl56 was performed. Transformation efficiencies were determined by monitoring the transient expression of *uid*A gene via histochemical GUS assay. Three to four weeks old calli were found to be more responsive to *Agrobacterium*-mediated transformation in Kızıltan cultures. However, four to five weeks old calli were found to be more responsive to Agrobacterium-mediated transformation in Bezostaja cultures. Different transformation protocols were used. It was found that MGL based and MMA based protocols could be used for Bezostaja and Kızıltan transformation, respectively. The highest GUS expression, 84%, was obtained from 28 weeks old calli produced from 5 weeks vernalised explants in Bezostaja cultures.

Keywords: Wheat immature inflorescence; Regeneration; *Agrobacterium tumefaciens*; GUS; Transient gene expression, Vernalization.

ÖZ

KIŞLIK BUĞDAYLARDA (*Kızıltan-91 and Bezostaja-01*) DOKU KÜLTÜRÜNÜN, REJENERASYONUN VE AGROBAKTERİUMA DAYALI TRANSFORMASYON PARAMETRELERİNİN OPTİMİZASYONU

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Bu çalışmada Kızıltan (*Triticum durum*) ve Bezostaja (*Triticum aestivum*) buğday çeşitlerinde olgunlaşmamış başak taslağına dayalı kültürlerin rejenerasyon parametrelerinin optimize edilmesi amaçlanmıştır. Kallus yaşının ve vernelizasyon süresinin rejenerasyon başarısına etkisi test edilmiştir. Optimum vernalizasyon süresini bulmak için bitkiler sırasıyla 1, 2, 3, 4, ve 5 hafta süreyle 4 ° C ye maruz bırakılmışlardır. Olgunlaşmamış başak taslağını içeren saplar aynı zamanda toplanmıştır. Kızıltan çeşitinde maximum % 79 inflorescence oluşturma oranına 4 hafta vernalize olan materyalde ulaşılırken Bezostaja da bu oran % 73 olup 5 hafta vernelize olan materyalden elde edilmiştir. İzole edilen olgunlaşmamış başak taslakları sırasıyla Kızıltan ve Bezostaja için 2 mg/L diklorofenoksiasetik asit ve picloram içeren kallus oluşum besiyerlerine konulmuştur. Kallus oluşturma yüzdesi Kızıltan ve Bezostaja için % 100 olarak bulunmuştur. Bu materyaller 6, 9, 12, ve 15 hafta karanlık inkubasyonundan sonra rejenerasyon ortamına alınmışlardır. Kallusların rejenerasyon kapasiteleri, çimlenme yüzdesi olarak belirlenmiştir ve veriler 4, 8, 12, ve 15 hafta rejenerasyon süresinden sonra

toplanmıştır. Kızıltanda en yüksek çimlenme yüzdesi % 69 olarak 4 hafta soğuğa maruz kalmış olan 6 haftalık kalluslardan elde edilmiştir. Çimlenme yüzdeleri 9 haftalık kalluslar için % 57.2, 12 haftalık kalluslar için % 37.6 ve 15 haftalık kalluslar için % 44.2 olmuştur. Bu, uzun süren karanlık inkübasyonunun kallus rejenerasyon kapasitesini azalttığını göstermektedir. Kızıltanın aksine Bezostajada değişik kallus yaşlarının rejenerasyon kapasitesi üzerine bir etkisi görülmemiştir ve en yüksek çimlenme yüzdesi ,% 27.4, 5 hafta soğuğa maruz kalmış 9 haftalık kalluslardan elde edilmiştir.

Rejenerasyon çalışmalarının yanısıra, Kızıltan ve Bezostaja kışlık buğday çeşitlerinde pALl56 ikili vektörünü içeren *Agrobacterium tumefaciens* AGLI suşu ile gen aktarımı parametreleri optimize edilmiştir. Transformasyon etkinliği *uid*A geninin geçici ifadesi takip edilerek histokimyasal GUS tayini ile belirlenmiştir. Kızıltanda üç-dört haftalık kallusların Agrobakteriuma dayalı transformasyona iyi cevap verdiği bulunmuştur. Bezostaja da ise dört-beş haftalık kallusların daha iyi cevap verdiği görülmüştür. Transformasyon sürecinde değişik protokoller kullanılmıştır.Kızıltan ve Bezostaja için sırasıyla MMA ve MGL tabanlı protokollerin kullanılabileceği görülmüştür. En yüksek GUS ifadesi, % 84, beş hafta soğuğa maruz kalmış materyalden elde edilen 28 günlük kalluslardan elde edilmiştir.

Anahtar kelimeler: Buğday, Başak taslağı, Rejenerasyon, *Agrobacterium tumefaciens,* GUS, Geçici gen ifadesi, Vernelizasyon.

to my wife

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2,4-D	2,4-dichlorophenoxyacetic acid
ANOVA	Analysis of variance
bp	Base pair
cv	Cultivated variety
EDTA	Ethylenediamine tetra acetic acid
GUS	β-glucuronidase
HCl	Hydrogen chloride
HMW-GS	High molecular weight glutenin subunits
LEA	Late Embryogenesis Abundant
MS	Murashige-Skoog basal salt medium
NaCl	Sodium chloride
NaOH	Sodium hydroxide
OD	Optical density
PEG	Polyethylene glycol
RFLP	Restriction fragment length polymorphism
rpm	Revolution per minute
spp.	Species
YEB	Yeast Extract Broth

CHAPTER I

INTRODUCTION

Wheat is the leading cereal grain produced, consumed and traded in the world. The cultivation of wheat is thought to have begun 8000 to 10000 years ago; and bread, leavened and unleavened has been stable food for humans throughout recorded history. The cultivation of wheat allowed the establishment of permanent settlement, fostering the development of civilization. Today, wheat also provides more food for human beings than any other source (Oleson, 1996).

1.1. Characteristics of Wheat

1.1.1. The Wheat Plant

Wheat is a monocotyledon of the Gramineae (Poaceae) family and belongs to *Triticum* genus. Wheat has a rather strong root system, with a number of fibrous and long roots growing to a depth of more than 1m in good conditions. The stem is cylindrical, erect, and sometimes solid, but more often hollow, subdivided into internodes. Leaves, distichous, are composed of a basal leaf sheath. Flowers are typically hermaphroditic, clustered in sessile that are layered upon each other to form spike. Normally wheat plant is self-fertile, even though outcrossing can reach 5 %, depending upon the variety and environmental conditions (Bozzini, 1988).

Wheat may exhibit either a winter or spring growth habit. Winter wheats are planted in the autumn and produce grain the following spring or summer. They require a vernalization period of temperatures near or slightly below freezing as

well as minimum accumulation of growing degree days and or length of daylight to convert from vegetative to reproductive growth. Accumulated growing degree days are the total number of day's average temperature above 0° C (Cook *et al.*, 1993). Vernalization response is common to fall-planted winter cereals and is survival mechanism to tolerate low temperatures. Wheat plants respond to vernalization by decreasing their time to flowering and there is no developmental response to vernalizing temperatures after flowering (Wang and Angel, 1998).

The seed, grain or kernel of wheat is a dry indehiscent fruit. The dorsal side (with respect to the spikelet axis) is smoothly rounded, while the ventral side has the deep crease. The embryo or germ is situated at the point of attachment of the spikelet axis, and the distal end has a brush of fine hairs. The embryo is made up of the scutellum, the plumule (shoot) and radicle (primary root). The scutellum is the region that secretes some of the enzymes involved in germination and absorbs the soluble sugars from breakdown of starch in the endosperm. Surrounding the endosperm is a metabolically active layer of cells or the aleurone layer, the testa or seed coat and pericarp or fruit coat. The coleoptile is well developed in the embryo, forming a thimble-shaped structure covering the leaf primordial and the shoot meristem (Kirby, 2002) (Figure 1.1).

1.1.2. Origin and Classification

Wheats 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 the Angiosperm class, the monocot cub-class, and the grass family. Within the grass family, wheat is a member of the tribe Triticeae and genus *Triticum* (Cook *et al.*, 1993).

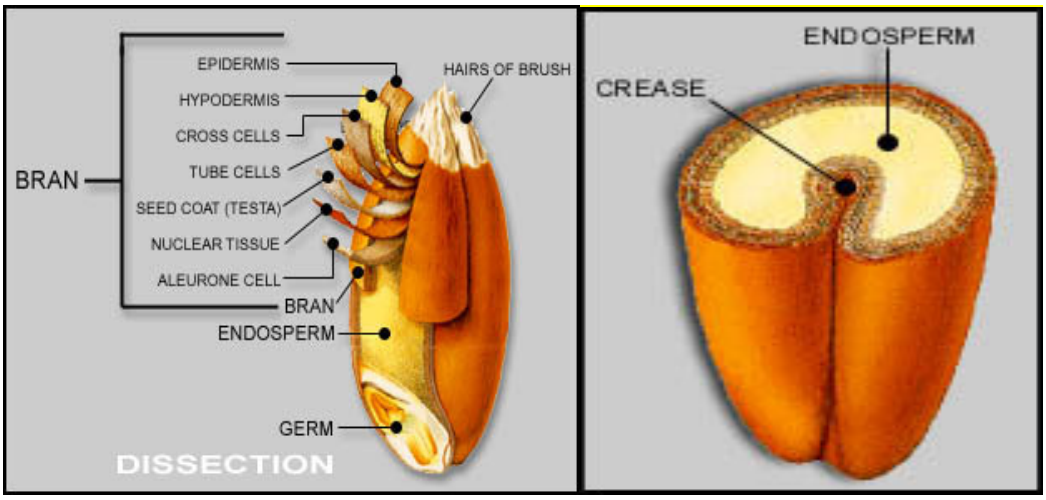

Figure 1.1. Structure of a Wheat kernel

The Fertile Crescent is considered the birth-place of cultivated wheats about 8 000 to 10 000 years ago. Pure stands of wild diploid einkorn and wild tetraploid emmer are found there and may have been harvested and cultivated as such. Recent genetic evidence indicates that einkorn wheat (*T. monococcum*) may have been domesticated from wild einkorn (*T. monococcum* ssp. *aegilopoides)* in the region of the Karacadag Mountains in southeast Turkey (Heun *et al.*, 1997). Both wild and cultivated einkorn seed remains have been excavated in the nearby archaeological sites dating from 7500 to 6200 BC. Diploid einkorn types of wheat are the earliest and the most primitive, while the hexaploids including the bread wheat, *Triticum aestevum,* constitute the most recent and latest step in the evolution of the wheat complex. Millions of years ago, the first hybridization event is thought to have occurred when the wild grass *Aegilops speltoides* crossed with the wild diploid wheat, *Triticum monococcum.* The resultant hybrid was the tetraploid emmer wheat, *Triticum dicoccum.* Domestication of emmer wheat led to the evolution of the durum wheat. Hybridization of tetraploid durum wheat, *Triticum turgidum* var. *durum* (2n=28, AABB) with the diploid wild goat grass, *Aegilops tauschii,* led to the origin and evolution of hexaploid wheat. Bread wheat

is thus an allohexaploid, containing three distinct but genetically related (homoeologous) copies each of the three originally independent haploid genomes, the A, B and D (Gill and Gill, 1994). Classification of wheat species are given in Table 1.1.

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	
	dicoccoides	Wild		
	dicoccum	Cultivated		
	durum	Cultivated		
	turgidum	Cultivated		
	paleocolchicum	Archaelogical		
	polonicum	Cultivated		
T. timopheevii	*armeniacum*	Wild	2n=28	AAGG
	timopheevi	Cultivated		
T. aestevum	*spelta*	Cultivated	2n=42	AABBDD
	macha	Cultivated		
	aestivum	Cultivated		
	compactum	Cultivated		
	sphaerococcum	Cultivated		

In Turkey, 10 different *Triticum* species, namely *T. boeoticum, T. monococcum L., T. timopheevii, T. dicoccoides, T. dicoccum, T. durum, T.turgidum, T. polonicum, T. carthlicum, T. aestivum* are grown naturally. *T. durum* Desf. (2n=48) and *T. aestivum* L. (2n=42) are the two most commonly cultivated wheats in Turkey today (Tan, 1985).

1.1.3. Genetic and cytogenetic characteristics of wheat

The basic number of chromosomes in 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 purposes 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 has a genome size of 10 billion bp of DNA organized into 14 pairs of chromosomes. The structure of A- and B-genome chromosomes of durum wheat is essentially identical to the corresponding homologues in bread wheat. Durum wheat is far less tolerant of aneuploidy than bread wheat, and monosomic analysis was difficult due to poor fertility of the monosomics. However, the developments of D-genome chromosome substitution lines and double ditelocentric stocks have greatly expedited chromosome and arm mapping of genes (Joppa, 1987). *Triticum aestivum*, bread wheat has a genome size of 16 billion base pairs (bp) of DNA organized into 21 pairs of chromosomes,

seven pairs belonging to each of the genomes A, B and D (Sears, 1954; Okamoto, 1962).

1.1.4. Uses

Wheat is a major diet component because of the wheat plant's agronomic adaptability, ease of grain storage and ease of converting grain into flour for making edible, palatable, interesting and satisfying foods. Dough produced from bread wheat flour differs from those made from other cereals in their unique viscoelastic properties (Orth and Shellenberger, 1988). Wheat is the most important source of carbohydrate in a majority of countries. Wheat starch is easily digested, as is most wheat protein. Wheat contains minerals, vitamins and fats (lipids), and with a small amount of animal or legume protein added is highly nutritious.

Roughly 90 to 95 percent of the wheat produced in the world is common wheat (*T. aestivum*), which is better known as hard wheat or soft wheat, depending on grain hardness. Wheat is utilized mainly as flour (whole grain or refined) for the production of a large variety of leavened and flat breads, and for the manufacture of a wide variety of other baking products. The rest is mostly durum wheat (*T. durum*), which is used to produce semolina (coarse flour), the main raw material of pasta making. Some durum wheat is milled into flour to manufacture medium-dense breads in Mediterranean and Middle Eastern countries and some into coarse durum grain grits used to produce *couscous* (cooked grits) in Arab countries(Curtis, 2002). Chemical components of the wheat kernel are shown in Table 1.2.
The types and utilizations of wheats are given in Table 1.3.

Table 1.2. Chemical components of the wheats (Adopted from http://www.professionalpasta.it/dir_1/flour_1.htm, 23.06.2005)

Chemicals	**Min.**	**Max.**
Protein (Nx5.7)	7.0	18.0
Ashes	1.5	2.0
Fats	1.5	2.0
Water (moisture)	8.0	18.0
Starch	60.0	68.0
Pentosans	6.2	8.0
Saccharose	0.2	0.6
Maltose	0.6	4.3
Cellulose	1.9	5.0

Besides being the major ingredient in most breads, rolls, crackers, cookies, biscuits, cakes, doughnuts, muffins, pancakes, waffles, noodles, pie crusts, ice cream cones, macaroni, spaghetti, puddings, pizza, and many prepared hot and cold breakfast foods, wheat it is also used in baby foods, and is a common thickener in soups, gravies, and sauces. Much of the wheat in the form of by-product of the flour milling industry is used for livestock and poultry feed. Wheat straw is used for livestock bedding (Gibson and Benson, 2002). About 6 % of wheat is used for industrial purposes which include the processing of wheat into starch and gluten for widely range of utilization in processed food products, the production of ethyl alcohol, plastics, varnishes, soaps, rubber and cosmetics (Oleson, 1994).

Table1.3. Types and utilizations of wheat

(Adopted from http://wbc.agr.state.mt.us/prodfacts/usf/usclass.html, 23.06.2005)

HARD RED WINTER WHEAT	Has wide range of protein content, good milling and baking characteristics. Used to produce bread, rolls and, to a lesser extent, sweet goods
HARD RED SPRING WHEAT	Contains the highest percentage of protein, making it excellent bread wheat with superior milling and baking characteristics.
SOFT RED WINTER WHEAT	Used for flat breads, cakes, pastries, and crackers.
HARD WHITE WHEAT	Closely related to red wheats, 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	Used in much the same way as Soft Red Winter (for bakery products other than bread). Contains low protein, but has high yielding. Used for production of flour for baking cakes, crackers, cookies, pastries, quick breads, muffins and snack foods.
DURUM WHEAT	Used to make semolina flour for pasta production. Common foods produced from durum wheat are macaroni, spaghetti, and similar products.

1.1.5. Production

Wheat is produced in 120 countries around the world and accounts for about 20 percent of the world's calorie supplies (Donald and Mielke, 2004). Wheat has the widest adaptation of all cereal crops. The heaviest concentration is in the temperate

zone of the northern hemisphere between the 30th and 60th latitudes, which includes the major grain growing areas of North America, Europe, Asia and North Africa (Oleson, 1994). According to United Nations Food and Agricultural Organization (FAO) statistics in 2004, top wheat producing nations are China, India, United States of America, Russian Federation, and France. According to FAO figures, the Worlds total wheat production was realized about 559 million tons in 2004 and Turkey had a 3.75 % world wheat production and rank ninth. The amount of wheat produced by top 10 producers and total amount of wheat produced in the world from 2000 to 2004 is given Table 1.4.

According to SIS data, in 2002, wheat was sown in 9.300.000 hectares of area, and 19.5 million tons of wheat was produced with en efficiency of 2101 kg/Ha in Turkey. Also in 2002, 77.8% of the total cultivatable land was used for wheat production. However, this amount is not sufficient for the national demand for wheat. In Union of Turkish Chambers of Agriculture 2004 Wheat Report, it was indicated that although utilization of wheat in developed countries is not so high, wheat based nutrition and high demand for wheat is common in Turkey and in countries where gross national income per capita is low. It was also mentioned that in Turkey 60% of daily calorie requirement was acquired from wheat. Although Turkey's wheat production was reached to 21 million tons in 2004, Turkey imported wheat in 2004 especially bread wheat and spent 276 million dollars for wheat import.

Table1.4. Top wheat producing nations (2000-2004), in terms of million tons

Country	2004	2003	2002	2001	2000
China	91.3	86.4	90.2	93.8	99.6
India	72.0	65.1	72.7	69.6	76.3
United States	59.0	63.8	44.0	53.2	60.7
Russia	42.2	34.0	50.6	46.9	34.4
France	39.6	30.4	38.9	31.5	37.3
Germany	25.3	19.2	20.8	22.8	21.6
Canada	24.4	23.5	16.1	20.5	26.5
Australia	22.5	24.9	10.0	24.8	22.1
Turkey	21.0	19.0	19.5	19.0	21.0
Pakistan	19.7	19.1	18.2	19.0	21.0
World Total	**559**	**569**	**588**	**585**	**592**

1.2. Improvement of Wheat

Wheat is second to rice in world importance. It provides protein, minerals, and vitamins as well as complex carbohydrates. It is the main food stable of 35% of the world's population and provides almost 20% of their total food calories. Global demand for wheat will grow faster than that for any other major crop and is forecasted to reach 1.1 billion tons in the year 2020, creating a challenge for molecular and conventional breeding (Kronstad, 1998). Today, the world's population is increasing at the most rapid rate ever. Two hundred people are being added to the planet every minute. It is forecast that by the year 2050, the world's population will double to nearly 12 billion people. To feed this population, these people will require a staggering increase in food production. In fact, it has been estimated that the world will need to produce more than twice as much food during the next 50 years as was produced since the beginning of agriculture 10 000 years ago (Hoisington *et al.* 2002).

1.2.1. Conventional Breeding

Conventional breeding has contributed to the genetic improvement of wheat and, since the early 1960s, has helped to increase grain yield and disease resistance. Subsequently, the focus of improvement has been directed towards reducing yield variability due to biotic and abiotic stresses (Pingali and Rajaram, 1999).

The conventional breeding techniques utilize processes of crossing, back crossing and selection. The main breeding schemes of wheat comprise the development of three types of wheat cultivars: pure lines, multilines, and hybrids. Pure lines are produced by cross-breeding followed by selection until the line is genetically uniform and its duration is usually eight to ten generations. Multilines are mixtures of pure lines. Hybrids are produced by either the cytoplasmic male-sterile method or by the chemical hybridization agent method. Pure-line crossbred cultivars are the most common ones (Cook *et al.*, 1993).

Wheat breeders have able to introduce desirable traits that increased the grain yield and minimize the crop loss. However, conventional breeding techniques which are based on processes of crossing, back crossing and selection, proved to be time consuming and, therefore, could hardly keep pace with the rapid co-evolution of pathogenic microorganisms and pests. The development of *in vitro* technologies have thus complemented the conventional methods of wheat breeding in generating genetic variability necessary for creating novel cultivars with desirable characters (Patnaik and Khurana, 2001).

Traditionally, genetic improvement in wheat is generated by using extensive crossing program and then systematically selection of useful new combinations (McIntosh RA, 1998; Bedo *et al.*, 1998). Although, with the help of the genetic variation present in wheat, plant breeders have tried for decades to improve the yield of wheat by using conventional methods of breeding, but their efforts were reaching plateau especially with respect to yield (Sahrawat *et al.*, 2003).

There are tremendous possibilities for wheat improvement with regards to increased pest and disease resistance, drought and freeze tolerance, better grain quality, and value-added traits. Solutions to some of these trait improvements lie outside the wheat gene pool and its relatives. Hence, these enhancements are beyond the scope of conventional breeding (Janakiraman *et al.*, 2002).

1.2.2. Biotechnological Approach

In recent years, biotechnology is emerging as one of the latest tools of agricultural research. In concert with traditional plant breeding practices, biotechnology is contributing towards the development of novel methods to genetically alter and control plant development, plant performance and plant products. The term biotechnology is composed of two words bio (Greek *bios*, means life) and technology (Greek *technologia,* means systematic treatment). Biotechnology involves the systematic application of biological processes for the beneficial use. One of the areas of plant biotechnology involves the delivery, integration and expression of defined genes into plant cells, which can be grown in artificial culture media to regenerate plants. Thus biotechnological approaches have the potential to complement conventional methods of breeding by reducing the time taken to produce cultivars with improved characteristics (Patnaik and Khurana, 2001).

Conventional breeding utilizes domestic crop cultivars and related genera as a source of genes for improvement of existing cultivars, and this process involves the transfer of a set of genes from the donor to the recipient. In contrast, biotechnological approaches can transfer defined genes from any organism, thereby increase the gene pool available for improvement. The improvement of wheat by biotechnological approaches primarily involves introduction of exogenous genes in a heritable manner, and secondarily, the availability of genes

that confer positive traits when genetically transferred into wheat (Patnaik and Khurana, 2001).

The genetic improvement of wheat has received considerable attention over the years from plant breeders with the purpose of increasing the grain yield and to minimize crop loss due to unfavorable environmental conditions, and attack by various pests and pathogens. In the early 60's, conventional breeding coupled with improved farm management practices led to a significant increase in world wheat production thereby lead to green revolution. Subsequently, the targets of genetic improvement shifted to reducing yield variability caused by various biotic and abiotic stresses and increasing the input-use efficiency (Pingali and Rajaram, 1999).

1.2.2.1. Tissue culture studies in wheat

A prerequisite for gene transfer by the commonly used techniques of *Agrobacterium*– mediated transformation or biolistics is a highly efficient method of producing undifferentiated callus tissue from a tissue explants and afterward triggering the regeneration of whole fertile plants. Wheat and monocots in general had been considered recalcitrant to such manipulations when compared to easily cultured species such as *Nicotiana tabacum* or *Daucus carota*. It was not until the 1980s that regeneration from callus culture could be routinely achieved. In wheat the explants that have been used to produce callus are those that are actively dividing or have only recently stopped dividing, which include nodes, leaf base, immature inflorescences, root tips, and scutellum (Mathias, 1990).

Until about 1980 there were only a few reports of reproducible plant regeneration from tissue cultures of the Gramineae. In most instances even the induction and maintenance of long-term callus cultures was found to be extremely difficult. In those species where plant regeneration was possible, it was limited to a few genotypes of little practical importance and number of plants formed was small.

Also, the regenerative capacity was often lost within the first few subcultures, after which calli either failed to grow or reverted to root forming cultures (Vasil, 1987).

1.2.2.1.1. General Characteristics of Plant Cell Culture

Genetic transformation of cereals including wheat largely depends on the ability of transformed tissues to proliferate on selection medium and subsequently regeneration of plants from transformed cells.

The rate of plant regeneration in tissue culture varies greatly from one species to another. Various cells, tissues and organs from numerous plant species can be cultured successfully to regenerate whole plants. There are several processes of plant regeneration for each species but usually only one type is most efficient (Ritchie and Hodges, 1993).

Plasticity and totipotency

Two concepts, plasticity and totipotency, are central to understanding plant cell culture and regeneration. Plants, due to their sessile nature and long life span, have developed a greater ability to endure extreme conditions and predation than have animals. Many of the processes involved in plant growth and development adapt to environmental conditions. This plasticity allows plants to alter their metabolism, growth and development to best suit their environment. Particularly important aspects of this adaptation, as far as plant tissue culture and regeneration are concerned, are the abilities to initiate cell division from almost any tissue of the plant and to regenerate lost organs or undergo different developmental pathways in response to particular stimuli (Slater *et al.*, 2003).

When plant cells and tissues are cultured *in vitro* they generally exhibit a very high degree of plasticity, which allows one type of tissue or organ to be initiated from another type. In this way, whole plants can be subsequently regenerated. This regeneration of whole organisms depends upon the concept that all plant cells can

give the correct stimuli, express the total genetic potential of the parent plant. 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, identifying the culture conditions and stimuli required to manifest this totipotency can be extremely difficult and it is still a largely empirical process (Slater *et al.*, 2003).

Plant Cell Culture Morphologies

All plant cell cultures are a heterogeneous mixture of cell types. Culture heterogeneity would be expected in organized cultures. As the presence of any morphogenesis in the culture require heterogeneity in the cell type. However, even in relatively homogeneous cultures, the microenvironment within different sized cell clusters could conceivably contribute to variation in cellular character. Additionally, there are at least two types of cells in any proliferating culture: meristematic and differentiated. Differentiated cells are characterized by being large, variably shaped, and highly vacuolated cells within a thin cytoplasm and inconspicuous nuclei. In contrast meristematic cells are characterized by having a small isodiametric shape, dense cytoplasm, little to no vacuolization, many starch grains, and large nuclei with prominent nucleoli (Ritchie and Hodges, 1993).

1.2.2.1.2. Factors affecting *in vitro* Culture

There are several factors affecting *in vitro* culture of wheat. The most important ones are the type of explants, genotype of the donor plant, growth conditions of the donor plant, and composition of the nutrient medium. In most species including cereals, plant regeneration depends on genotype (Maddock *et al.*, 1983; Mathias and Simpson, 1986; Fennell et al, 1996, Özgen et al, 1998) the type of explant (Ozias-Akins and Vasil, 1982; Maddock *et al.*, 1983; Zhang and Seilleur, 1987; Redway *et al.*, 1990) and media composition (Mathias and Simpson, 1986; Elena and Ginzo, 1988; Fennell *et al.*, 1996; He *et al.*, 1998). Application of some

methods are, in many cases, restricted to only a particular set of genotypes, types of explants or culture media.

Sears and Deckard, in 1982, investigated the genotypic differences in immature embryo based callus cultures. They have found that for most of genotypes, the degree of cellular organization and shoot meristems can be controlled by manipulation of 2,4-D concentration which indicates variation in tolerance to 2,4-D amongst genotypes. Also, Maddock *et al.* (1983) evaluated plant regeneration from immature embryo and inflorescence tissues of 25 wheat cultivars and they reported clear differences in morphogenic capacities of genotypes. Vasil (1987) has indicated 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 is also suggested by Vasil that when suitable explants at defined developmental stages are excised from plants and cultured under optimal conditions with appropriate amount of plant growth regulators, even recalcitrant plants or genotypes can be induced for morphogenesis.

According to Qureshi and co-workers (1989), composition of the nutrient medium and phytohormones are very important for plant regeneration (Pellegrineschi et al, 2004). The last important point which effects the regeneration of wheat is growth conditions of donor plant (Vasil, 1982; Vasil, 1987; Harvey et al, 1999; Delporte et al, 2001).

1.2.2.1.3. Characteristics of Embryogenic Cultures

Embryogenic callus cultures can be obtained most readily from immature embryos, young inflorescences, and leaves. In embryos the callus originates from peripheral cells in specific and predictable regions of scutellum (Vasil, 1982; Lu and Vasil, 1985), in inflorescences from the floral meristems or occasionally also from the peripheral ground tissue around vascular bundles (Botti and Vasil, 1984),

and in leaves from cells of the lower epidermis and mesophyll near vascular bundles (Wernicke and Milkovits, 1984; Lu and Vasil, 1981; Conger *et al.*, 1983).

At the beginning of excision and culture of explant in the presence of 2,4-D, embryogenic competence is expressed by 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 cultures by continued divisions in embryogenic cells and in active meristematic zones formed in proliferating tissues. Lowering of 2,4-D levels result 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 disappears (Vasil and Vasil, 1982).

This irreversible process of 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 the 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). For the maintenance of the embryogenic potential, and to ensure that plant regeneration is solely via somatic embryogenesis, it is essential to visually select and transfer embryogenic calli during subcultures (Vasil, 1987).

The embryogenic calli are characteristically compact, rather organized, and white to pale yellow in color. Initially such calli exhibit slow rates of growth, though some species fast growing calli may be obtained. In a few instances a soft, non-embryogenic callus is formed initially, and the characteristic white and compact

embryogenic callus appears only later in localized areas (Vasil and Vasil, 1982; Green and Rhodes, 1982).

1.2.2.1.4. Studies on Wheat Regeneration Systems

A highly efficient and reproducible *in vitro* regeneration system is an absolute prerequisite for producing transgenic plants. Particularly in the cereals routine application of molecular improvement independent of the chosen method of transformation is still impeded by the lack of readily available highly efficient and long-term regenerable cell and tissue culture systems (Sharma *et al.*, 2004).

Plant regeneration in tissue cultures can follow two different pathways. These are organogenesis involving the development of axillary buds following inhibition of apical dominance and somatic embryogenesis (Vasil, 1987).

The formation of callus from an explant roughly contains 3 stages: induction, cell division, and differentiation. During induction phase, the metabolism is prepared for cell division. At the actively cell division phase, the cells of the explant are reverted to meristematic or dedifferentiated state. Third phase is the appearance of cellular differentiation and expression of certain metabolic pathways (Dodds and Roberts, 1985).

Somatic embryogenesis and organogenesis

In somatic (asexual) embryogenesis, embryo-like structures, which can develop into whole plants in a way analogous to zygotic embryos, are formed from somatic tissues. These somatic embryos can be produced either directly or indirectly. In direct somatic embryogenesis, the embryo is formed directly from a cell or small group of cells without the production of an intervening callus. Though common from some tissues (usually reproductive tissues such as the nucellus, styles or pollen), direct somatic embryogenesis is generally rare in comparison with indirect

somatic embryogenesis. In indirect somatic embryogenesis, callus is first produced from the explant. Embryos can then be produced from the callus tissue or from a cell suspension produced from that callus (Slater *et al.*, 2003).

Germination of somatic embryos is usually induced on hormone-free culture medium or medium that contains low levels of an auxin or low levels of both an auxin and a cytokinin. Under these conditions, the somatic embryos which are developmentally capable of germinating do so without maturing. Unlike mature zygotic embryos, which germinate vigorously into sizeable plants within several days, germinating somatic embryos generally lack vigor and may require many days to develop into healthy, rapidly growing plants (Carman, 1995).

Organogenesis

Somatic embryogenesis relies on plant regeneration through a process analogous to zygotic embryo germination. Organogenesis relies on the production of organs, either directly from an explant or from a callus culture. There are three methods of plant regeneration via organogenesis. The first two methods depend on adventitious organs arising either from a callus culture or directly from an explant. Alternatively, axillary bud formation and growth can also be used to regenerate whole plants from some types of tissue culture (Slater *et al.*, 2003).

Various explants sources, such as whole seeds, mature and immature embryos, isolated scutellum, immature inflorescence, immature leaf, monocotyl, apical meristem, coleoptilar node, and root have been used in attempts to establish regenerable tissue culture of wheat (Rakszegi *et al.*, 2001). Most widely used explants are immature embryos, immature inflorescences, and mature embryos and an overview of the studies using these explants is given below.

Immature embryos

In cereals, immature embryos are considered the most responsive explant in culture because of their ability to produce readily embryogenic callus and subsequently large number of plants.

Complete plantlets were first regenerated from immature embryos of wheat by Shimada (1978). Later, Shimada and Yamada (1979) optimized various factors, such as age and size of the embryos and auxin concentration for callus induction and found that embryos isolated 14 days after anthesis induced high intensity of green spots formation.

In 1982, Sears and Deckard examined immature embryos obtained from 39 genotypes of winter wheat for potential use of tissue culture. They observed variability among the wheat genotypes tested for callus induction, regenerable callus formation, response to subculture, and plant regeneration potential.

Ozias-Akins and Vasil (1983) observed somatic embryos in tissue cultures derived from the scutellum of immature embryos of 3 different *T. aestivum* cultivars. They reported that the frequency of white embryogenic callus formation could be increased to at least 30% on media with double concentration of MS inorganic salts.

Mathias and Simpson (1986) investigated the effect of interaction of genotype and culture medium on the initiation of callus from immature embryos and subsequent plant regeneration by using eight hexaploid wheat lines. They observed that primordial and shoot development was promoted by coconut milk in some line but the others not. They concluded that the genotype clearly has more effect on the response of calli than the presence of complex organic additives in the media.

In 1988, Bennici and his coworkers investigated callus formation and plantlet regeneration from immature *T. durum* embryos. They found that the ability to form callus and the degree of morphogenetic processes varied with different hormonal treatments used and with the age of the embryos.

Borrelli and his colleagues (1991) tested five durum wheat cultivars for their *in vitro* response. They obtained plantlets as a result of somatic embryogenesis of long term selective subculture of immature embryo derived embryogenic calli.

Felföldi and Purnhauser (1992) investigated the induction of regenerating callus cultures from immature embryos of 44 wheat and 3 triticale cultivars. They reported that the frequency of callus induction generally did not vary significantly among genotypes. However, the frequency of regeneration could differ among genotypes.

In 1996, Fennell and his colleagues have regenerated plantlets from immature embryos derived calli of 48 elite bread wheat cultivars. They achieved plant regeneration by using IAA and BAP in nutrient medium. Also they found that percentage regeneration varied widely with both genotype and initiation medium used.

In 1998, Machii and coworkers screened wheat genotypes for high callus induction and regeneration capability from anther and immature embryo cultures. They reported that calli derived from immature embryos have more regeneration capacity than the calli derived from anther. And also they declared that regeneration callus inductions highly depend on genotype.

Fernandez and his colleagues (1999) have examined the embryogenic response of immature embryo cultures of durum wheat. They have reported that the addition of 1 mg/L $AgNO_3$ enhanced the induction of direct somatic embryogenesis up to 22

fold, affecting both the percentage of embryogenic explants and the number of somatic embryos per explant.

In 1999, Arzani and Mirodjagh have investigated the response of durum wheat cultivars to immature embryo cultures, callus induction and *in vitro* salt stress. They observed that there were significant differences among eight cultivars for the potential of regeneration from immature embryo, relative fresh weight growth of callus and callus necrosis percent. Also their result show that the relative fresh growth rate of callus decreased as the concentration of NaCl increased.

Gonzalez and colleagues (2001) were evaluated influence of genotype and culture medium on callus formation and plant regeneration from immature embryos of 12 different durum wheat cultivars. They have reported that the regeneration of plantlets was higher from compact than from soft calli, with a strong dependence on genotype and type of induction medium utilized.

In 2001, Bohorova and coworkers have examined the regeneration potential of 25 durum wheat and five triticale cultivars. They found that regeneration rates varied widely with both genotype and initiation medium, with values ranging from no regeneration to 100% regeneration.

Khanna and Daggard (2001) has reported that the regeneration potential of ageing calli initiated from isolated scutella of immature embryos was increased in nine elite Australian cultivars by using spermidine and dehydration stress. Their results showed that some calli which had undergone 16 h of dehydration stress up to 12 weeks old had shown improved regeneration.

In 2003, Przetakiewicz and colleagues have investigated the effect of auxin on plant regeneration of wheat, barley and triticale. They have compared the efficiency of plant development on two regeneration media, with and without growth regulators. They have reported that mean number of plantlets regenerating

per explant differed significantly depending on the type of auxin in inducing media, the type of regenerating media as well as cultivar.

Pellegrineschi and colleagues (2004) have obtained optimal callus induction and plant regeneration in bread and durum wheat by manipulating NaCl concentration in the induction medium. Callus yield and regeneration frequencies were higher in durum wheat embryos that were incubated in media containing 2 mg/L 2,4-D and 2 mg/L NaCl.

However, since immature embryos are not readily available all year around, a more convenient explant that has this ability; i.e. mature embryos, also used by many researchers.

Mature embryos

Heyser and colleagues (1985) have investigated long-term, high frequency plant regeneration and the induction of somatic embryogenesis in callus cultures of *Triticum aestivum* using mature and immature embryos. They found that the relative amounts of embryogenic and non-embryogenic callus in mature and immature embryos could be significantly altered by different 2,4-D concentrations in the medium.

Özgen and coworkers (1996) have reported callus induction and plant regeneration from immature and mature embryos of winter durum wheat cultivars. They declared that callus induction rate and regeneration capacity of callus were independent of each other. Also they observed that mature embryos have a low frequency of callus induction but a high regeneration capacity and variability among the wheat genotypes tested for various culture responses in both explant cultures.

In 1998, Özgen and colleagues cultured mature and immature embryos of 12 common winter wheat genotypes and compared their regeneration response. They confirmed that callus induction rate, regeneration capacity of callus and number of plants regenerated were independent of each other. They concluded that callus responses of mature embryo culture may be higher than those of immature embryo culture.

Delporte and coworkers (2001) developed a wheat regeneration system using mature embryo fragments of wheat. They used thin mature embryo fragments as explants to initiate embryogenic calli on solid medium supplemented with 10 µM 2,4-D. They reached the highest embryogenic calli induction rate of 47% when 2,4-D suppressed after a 3-4 week induction period.

In 2001, Özgen and his colleagues investigated cytoplasmic effects on the tissue culture response of callus derived mature embryos from four different winter wheat cultivars. They found significant reciprocal differences for tissue culture response of callus, indicating that a cytoplasmic effect may be involved.

Zale and coworkers (2004) compared the behavior of a diverse set of wheat genotypes in their tissue culture response using mature embryos. They found significant differences in plant regeneration, culture efficiency, and regeneration capacity when mature embryos of 47 wheat cultivars, breeding lines, and the common wheat progenitors were compared. Also they declared that there was no significant correlation between total callus induction and regeneration capacity or culture efficiency indicating that these variables were not related.

Immature inflorescences

Immature inflorescences are an alternative explant for plant regeneration studies. Advantages of using inflorescence tissue versus scutella are that explants are harvested from younger plants reducing greenhouse or growth chamber requirements.

In 1982, Ozias-Akins and Vasil reported tissue cultures of *Triticum aestivum* initiated from young inflorescences and immature embryos possessed the potential for regeneration of whole plants. They declared that both a friable and a compact type of callus were produced on MS medium supplemented with 2 mg/L 2,4-D. The friable callus contained meristematic centers in which the peripheral cells halted dividing, elongated, and could be easily separated. They stated that embryogenic callus from inflorescence tissue and from immature embryo have morphological similarities.

Maddock and co-workers (1983) have obtained regenerated wheat plats using cultured immature embryos and inflorescences of 25 cultivars of wheat. They compared morphogenetic capacities of 25 different cultivars of spring and winter wheat, and clear differences were found between genotypes. They found that the ranges in response in cultured inflorescences are from 33% to 100%.

In 1990, Redway and colleagues have identified the callus types in commercial wheat cultivars. They reported that there were no significant differences in the response of calli derived from embryos and immature inflorescences cultured on different initiation media. Also, the shoot-forming capacities of the cultivars were not significantly different. Also they observed two types of embryogenic calli: off-white, compact, and nodular callus and a white compact callus.

In 1995, Sharma and coworkers have compared developmental stages of inflorescence for high frequency plant regeneration in *Triticum aestivum* and

Triticum durum. They found that 0.5 and 1.0 cm long inflorescences responded best in forming callus as well as plantlets at a very high frequency. They observed variation in callus forming response among genotypes.

Barro and coworkers (1999) have tried to optimize a medium for efficient somatic embryogenesis and plant regeneration from immature inflorescences and immature scutella of elite cultivars of wheat, barley and tritordeum. They reported that for wheat and tritordeum inflorescences, regeneration from embryogenic calluses induced on medium supplemented with picloram was almost twice as efficient as regeneration from cultures induced on 2,4-D. Also, they observed that the addition of Zeatin to regeneration media had a positive effect on regeneration. They concluded that embryogenic capacity from inflorescences was higher than from immature scutella.

In 2000, Caswell and colleagues have developed an efficient method for *in vitro* regeneration from immature inflorescence explants of Canadian wheat cultivars. They used two different medium for callus induction and regeneration. They reported that the percentage of explants which produced shoots was significantly higher on medium containing 250 mg/L NH_4NO_3 and maltose as a carbon source than medium containing 1650 mg/L NH_4NO_3 and sucrose as a carbon source, regardless of the size range of the immature inflorescences.

Benkirane and coworkers (2000) have reported the somatic embryogenesis and plant regeneration from fragments of immature inflorescences and coleoptiles of durum wheat. They obtained the highest frequencies of embryogenic callus, as 100% for most cultivars, and regeneration from 0.5 or 1 cm long inflorescences fragments incubated on media containing 6.8 mM of 2,4-D. Also, they observed for both inflorescence and coleoptile explants a genotype effect on callus induction and plant regeneration.

He and Lazzeri (2001) used scutellum and inflorescence explants of four genotypes of durum wheat to define culture conditions to obtain high frequencies of embryogenesis and plant regeneration. They found that scutellum cultures gave higher frequencies of embryogenesis and regeneration than inflorescence cultures. Also, they observed that picloram significantly increased the frequency of plant regeneration from both explants. They obtained the most plantlets from medium containing 4 mg/L picloram for immature inflorescences.

Durusu (2001) investigated the embryogenic potentials of Turkish wheat cultivars from immature embryo and inflorescence explants. In this study, the regeneration potential of eight Turkish wheat cultivars was compared and immature inflorescences were found to have higher regeneration capacities than immature embryos.

Demirbaş (2004) was optimized regeneration parameters from immature inflorescences of Turkish bread wheat cultivar Yüreğir. Also, she determined the embryogenic capacity and regeneration potential of different regions of inflorescence tissues.

1.2.2.2. Transformation of Wheat

Despite its global importance, wheat was the last major cereal to be genetically transformed. It is only a decade since the recalcitrance of this crop to *in vitro* culture was overcame (Jones, 2005). The first reports of fertile adult transgenic wheat plants were published (Vasil *et al.*, 1992, 1993; Weeks *et al.*, 1993; Becker *et al.*, 1994; Nehra *et al.*, 1994). Since then, other key advances have been made, including the development of *Agrobacterium*-mediated transformation, the reduction of genotype dependency and improvements in transformation efficiency. Successful wheat transformation is depends on a number of factors such as the genotype, growth conditions of the donor plants, tissue culture and transformation protocol. Wheat transformation, like all plant genetic engineering protocols, can be

separated into three basic steps: target tissue preparation, gene introduction, and selection and recovery of transgenic plants (Janakiraman *et al.*, 2002)

1.2.2.2.1. Explants Types Used In Wheat Transformation

Adult, fertile transgenic wheat plants can be regenerated from only a few target cell-types, although the choice is wider for transient expression studies or if transformed but non-regenerable cell lines are the final product. Only two wheat explant tissues are currently used routinely for making transgenic plants; the immature inflorescence and the scutellum of immature zygotic embryos (Jones, 2005). Other tissues that have been used for *in vitro* regeneration of plants but are not yet capable of reliably producing fertile adult transgenic wheat plants include shoot meristems (Ahmad *et al.*, 2002), leaf bases (Wang and Wei, 2004), microspores (Folling and Olesen, 2001; Ingram *et al.*, 1999; Kunz *et al.*, 2000; Liu *et al.*, 2002) and mature seeds (Londsdale *et al*., 1990; Özgen *et al.*, 1998; Zale *et al.*, 2004).

The most commonly used explant for wheat transformation is the immature scutellum, a specialized tissue that forms part of the seed embryo. It is amenable to both biolistics and *Agrobacterium*-mediated DNA delivery methods and can be readily induced to form embryogenic callus (Jones, 2005). The optimal stage of caryopsis development for embryo isolation can differ between genotypes but is around 11–16 days post anthesis (Pastori *et al.*, 2001). A slight improvement in transformation was observed when scutella were isolated from donor plants that had themselves been regenerated via embryogenesis and tissue culture (Harvey *et al.*, 1999).This improvement in overall transformation rates was attributed to an improvement in the consistency of donor plants resulting in fewer failed experiments.

More recently, immature inflorescences have been reported as an alternative source of explant for stable transformation of durum wheat (He and Lazzeri, 1998;

Lamacchia *et al.*, 2001), tritordeum (Barcelo *et al.*, 1994; He *et al.*, 2001) and bread wheat (Rasco-Gaunt and Barcelo, 1999; Sparks *et al.*, 2001). This explant has also shown good expression of T-DNA delivered genes after *Agrobacterium* co-cultivation (Amoah *et al.*, 2001). Advantages of the immature inflorescence system are that they are easier to isolate and are harvested from much younger plants allowing more efficient use of space in donor-plant growth facilities. However, their tissue culture response is more genotype-specific than that of immature scutella, with some varieties being particularly unresponsive (Rasco-Gaunt and Barcelo, 1999).

1.2.2.2.2. Techniques Used in Wheat Transformation

Many DNA-transfer methods have been tried with varying degrees of success including electroporation, micro-injection, silicon carbide fibres, polyethylene glycol and laser-mediated uptake, but two methods now predominate: transformation via particle bombardment and via *Agrobacterium* (Jones, 2005).

1.2.2.2.2.1. Electroporation

Electroporation is a technique that utilizes a high intensity electric pulse to create transient pores in the cell membrane thereby facilitating the uptake of macromolecules like DNA.

Ou-Lee *et al.* (1986) reported the expression of the bacterial chloramphenicol acetyl transferase (*cat*) gene in three important graminaceous plants, *i.e.* rice, sorghum and wheat. The survival percentage of protoplasts after electroporation depended largely on the tissue of origin.

Zagmout and Trolinder (1993) optimised the conditions for a significant increase in *gus* activity which included the protoplast source, the promoter, the polyethylene glycol (PEG), as well as, electroporation parameters especially the

electric field strength, preincubation with the plasmid, and recovery period on ice after the electric pulse.

Zhou *et al.*, (1993) reported stable transformation by electroporation using plasmid pBARGUS into protoplasts isolated from cell suspension initiated from an anther-derived callus. Also He *et al.* (1994) reported stable transformation of protoplasts by electroporation employing the *bar* gene as the selectable marker.

1.2.2.2.2.2. Microprojectile Bombardment

Success in genetic transformation of cereals was difficult to achieve and often limited to transient gene expression because of the lack of suitable regenerative systems and incapability of *Agrobacterium* to infect cereal tissues. This is the main reason that the method of introducing DNA into cells by physical means (microprojectile bombardment) was developed to overcome the biological limitations of *Agrobacterium* and difficulties associated with plant regeneration from protoplasts (Sahrawat *et al.*, 2003).

Particle or microprojectile bombardment (also called biolistics) involves the adsorption of plasmid or linear forms of naked DNA onto the 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). Initial studies on particle bombardment as a gene delivery method achieved transient expression of *gus* gene following bombardment of cell suspensions (Wang *et al.* 1988), leaf bases and apical tissues (Oard *et al.* 1990), immature embryos (Chibbar *et al.* 1991). Wheat is utilized as a target tissue for the first time by Londsdale and co-workers (1990) who transformed mature wheat embryos and showed transient expression of the *uid*A gene. Vasil *et al.* (1991) obtained stably transformed callus lines that expressed all the marker genes tested (*gus*, *nptI* and *EPSPS*). First successful generation of transgenic wheat plants was reported by Vasil *et al.* (1992) by particle bombardment of plasmid vector pBARGUS into cells of type-C,

long-term regenerable embryogenic callus derived from immature embryo and this report considered being a milestone in transgenic research.

The first successful transformation of durum wheat was reported by Bommineni *et al.* (1997) by particle bombardment of isolated scutella. Particle bombardment of explants like pollen embryos (Shimada *et al.* 1991), microspore-derived embryos (Loeb and Reynolds, 1994; Ingram *et al.* 1999) resulted in successful introduction of transgenes as evidenced by transient expression of *gus* gene, however regeneration of plantlets from these explants has not been reported (Patnaik *et al.* 2001).

The effects of various bombardment parameters like amount of plasmid DNA, spermidine concentration, acceleration and vacuum pressure, osmotic pretreatment of target tissues on gene delivery into wheat tissues have been investigated in detail by Rasco-Gaunt *et al.* (1999) and most of the studies have found no clear correlation between transient expression and stable transformation. In later studies, it has been shown that the transgenes introduced by biolistic approach display a considerable degree of stability in integration and expression in subsequent generations (Altpeter *et al.*, 1996; Srivastava *et al.*, 1996).

Öktem and co-workers (1999) transformed two Turkish wheat cultivars by particle bombardment of mature embryos and they observed that almost 80% of the bombarded embryos expressed the transferred GUS gene through blue colour formation on the embryos.

Genotype and age of the donor plants are two important factors in order to achieve successful transformation of wheat. Varshney and Altpeter (2001) have reported that there is significant variability both in regeneration and transformation frequency between 38 German wheat cultivars using immature embryos.

Pastori *et al.* (2001) transformed two elite wheat varieties by particle bombardment of scutella isolated from immature embryos and observed a strong correlation between transformation frequency and the age of the wheat donor plant. They were able to increase mean transformation frequency from 0.7 to 5% by using immature embryos from young plants.

Rasco-Gaunt *et al.* (2001) transformed ten European wheat varieties at efficiencies ranging from 1 to 17% (mean 4% across varieties) by optimizing particle bombardment and tissue culture procedure. Among parameters tested, authors found that selection of transformed scutella at high sucrose level (9%) enhanced somatic embryogenesis, shoot induction and consequently stable transformation efficiency.

Patnaik and Khurana (2003) obtained transgenic wheat plant using biolistic method from mature embryo derived calli and immature embryo derived calli successfully. They confirmed the transgenes activities in T_0 and T_1 generation plants using southern analysis.

1.2.2.2.2.3. *Agrobacterium*-mediated transformation

Agrobacterium tumefaciens is a soil bacterium that has been implicated in gall formation at the wound sites of many dicotyledonous plants. The tumour-inducing capability is due to the presence of a large Ti (tumour-inducing) plasmid in virulent strains of *Agrobacterium.* The important requirements for *Agrobacterium*-mediated transformation firstly include the production of some active compounds like acetosyringone by the explants in order to induce the *vir* genes present on the Ti plasmid and then the induced agrobacteria must have access to competent plant cells that are capable of regenerating adventitious shoots or somatic embryos at a reasonable frequency. The induction of *vir* genes which is necessary for gene transfer has been achieved by wounding and also by using chemical inducers like acetosyringone.

Agrobacterium-based DNA transfer system offers many unique advantages in plant transformation:

I. The simplicity of *Agrobacterium* gene transfer makes it a 'poor man's vector.

II. The ability to transfer long stretches of T-DNA

III. High co-expression of multiple introduced genes

These advantages allow production of plants with simple integration pattern along with high frequency of stable genomic integration and single-low copy number of the intact transgene (Rakszegi *et al.,* 2001; Janakiraman *et al.,* 2001; Sahrawat *et al.*, 2003;Veluthambi *et al.*, 2003). However, for monocotyledonous plants *Agrobacterium* mediated gene delivery is still far from a routine technique.

The first reports of successful *Agrobacterium*-mediated transformation of wheat were those of Hess *et al.* (1990), which involved pipetting *Agrobacterium* onto the spikelets to introduce Kanamycin resistance.

Mooney and colleagues (1991) reported the first transformation of wheat embryo cells co-cultivated with *Agrobacterium tumefaciens* and for the first time demonstrated that wounding is not necessary for adherence of bacteria to explants of wheat. Mooney suggested that the increase in adherence of bacteria at the wound site caused by mechanical and enzymatic treatments. Later, in 1992, Chen and Dale reported a higher frequency of infection in exposed apical meristems of dry wheat seeds as compared to intact seeds.

Chan and coworkers (1993) produced a few transgenic rice plants by inoculating immature embryos with *A. tumefaciens*. Although they showed the inheritance of transferred DNA to progeny plants but their analysis was based only on one transformed plant. In 1994, Hiei and colleagues demonstrated irrefutable evidences for genetic transformation of Japonica rice mediated by A. tumefaciens and

developed a method for efficient production of fertile transgenic rice plants for the first time. Their evidence was based on molecular and genetic analysis of large number of transgenic plants and also on the analysis of the sequence of T-DNA junction in transgenic plants. Although they utilize super binary vectors, their studies contributed to better understanding of various parameters required for successful transformation of cereals via *Agrobacterium.*

Mahalakshmi and Khurana (1995) tested the suitability of various wheat explants for *Agrobacterium* mediated gene delivery and reported an increase in transient expression of GUS gene in mature seeds subjected to mechanical wounding by abrasion as compared to intact seeds.

In genetic transformation of cereals, another break through came in 1997, when Tingay and his coworkers used non-super virulent strain and reported successful transformation of barley. A phenolic compound 'acetosyringone' which is known to induce expression of virulence (*vir*) genes located on the Ti-plasmid played a major role in the success of their study.

The stable transformation of wheat by *Agrobacterium*-mediated co-cultivation was reported by Cheng and his coworkers (1997) by using immature embryos for the first time. Also they demonstrated the successful transmission of the transgene to the next generation. This study developed a system for the production of transgenic plants within a total time of 2.5 to 3 months by co-cultivating freshly isolated immature embryos, precultured immature embryos and embryogenic calli.

Amoah and colleagues (2001) have investigated factors influencing *Agrobacterium*-mediated transient expression of *gus* gene in wheat inflorescence tissue. During transformation studies, they used *Agrobacterium tumefaciens* strain AGL harboring the binary vector pAL156. They studied the effects various factors on delivery and transient expression of *gus* gene including the duration of precultured, vacuum infiltration, the effect of sonication treatments and

Agrobacterium cell density. They obtained optimal T-DNA delivery from inflorescence tissues precultured for 21 days and sonicated.

In 2003, Wu and colleagues have investigated factors effecting successful *Agrobacterium*-mediated genetic transformation. They used immature embryos from range of wheat varieties and the *Agrobacterium* strain AGL I harboring plasmid pAL156. They report some factor effecting T-DNA delivery efficiency, such as; embryo size, duration of pre-culture, inoculation and co-cultivation, and presence of acetosyringone in media.

Haliloğlu and Baenziger (2003) developed an efficient and reproducible *Agrobacterium tumefaciens*-mediated system for wheat transformation and they produced genetically transformed wheat plants using immature embryos as the explant. They demonstrated stable integration, expression and inheritance of transgenes by molecular and genetic analysis of transformants.

In 2003, Khanna and Daggard transformed immature embryo derived calli of spring wheat using *Agrobacterium tumefaciens* strain LBA4404. Their results suggest that two important factors that could lead the improvement in transformation frequencies of cereals like wheat are: the use of superbinary vectors and modification of the polyamine ratio in the regeneration medium.

Przetakiewicz and colleagues (2004) performed *Agrobacterium* mediated transformation of wheat by using three combinations of *Agrobacterium tumefaciens* strains and vectors and selected Polish wheat cultivars. They obtained the highest selection rate from EHA101 on kanamycin containing medium as 12.6%. They confirmed the transgene integration via southern blot analysis.

1.2.2.2.3. Agronomically important genes transferred to wheat

Recent advances in transformation technology have resulted in the routine production of transgenic wheat plants for the introduction of not only marker genes but also agronomically important genes for quality improvement, male sterility, transposon tagging, resistance to drought stress, resistance against fungal pathogen and insect resistance. Agronomically important genes incorporated into wheat via particle bombardment are demonstrated in Table 1.5.

Table 1.5. Agronomically important genes transferred into wheat (Adopted from Sahrawat *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
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

Table 1.5. Continued

IE	*O. sativa*	Thaumatin-like 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
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	*Galanthus-nivalis 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

Table 1.5. Continued

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
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*	Phytase-encoding gene (*PhyA*)	*bar*	Accumulation of phytage in transgenic seeds	Brinch-Pedersen *et al.*, 2000
IE	*H. vulgare* L.	Ribosome-inactivating 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 1.5. 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	*T. aestivum* L. (soft wheat)	Protein puroindoline (PinB-D1a)	*Bar*	Increased friabilin levels and decreased kernel hardness	Beecher *et al.*, 2002
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.

1.2. Aim of the study

The main objective of this study is the optimization of immature inflorescence based regeneration and transformation systems for two winter wheat cultivars (Kızıltan-91 and Bezostaja-01). For these reasons, the following approaches have been considered:

i. determination of effect of vernalization time on inflorescence formation,
ii. determination of the effect of callus age on regeneration,
iii. determination of correlation of vernalization time with other parameters such as regeneration frequency, number of shoots, number of spikes, weight and number of seed,
iv. optimisation of *Agrobacterium*-mediated transformation parameters based on callus age and vernalization time.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Materials

In this study, immature inflorescences of winter durum wheat *Triticum durum* cultivar Kızıltan-91 and winter bread wheat *Triticum aestivum* cultivar Bezostaja-01 were used. The seeds were obtained from Agricultural Research Institute. Additional information on cultivars were given in Appendix A.

2.1.2. Chemicals

The chemicals used in this study were obtained from Duchefa (Haarlem, The Nederland), Sigma Chemical Company (N.Y., USA), and Merck Chemical Company (Desenhoen, Deutschland). All of the solutions were prepared by using distilled water.

2.1.3. Plant Tissue Culture Media

2.1.3.1. Induction and Maintenance Media

In Kızıltan-91 tissue culture studies, MS based media containing MS basal salts (Murashige and Skoog, 1962), 3 % sucrose and 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) were used for callus induction and maintenance. For Bezostaja-01

tissue culture studies, three different MS based medium namely I_1, I_2 and MS_2 as callus induction and maintenance media were used (Table 2.1). The medium composition of MS is given in Appendix B. All media were solidified with 0.28 % Phytagel and their pH was adjusted to 5.8 with NaOH or HCl and then sterilized by autoclaving at 121 ° C for 20 minutes.

2.1.3.2. Regeneration Media

In Kızıltan-91 tissue culture studies, half strength MS basal salts and 3 % sucrose were used as regeneration medium. For Bezostaja-1 cultivar, R_1, R_2 and MS/2 were used as regeneration media (Table 2.1).

2.1.3.3. Transformation Media

During transformation studies, MMA and IMM media were used for bacterial inoculation of calli, and were for elimination of bacteria after transformation by addition of 500 mg/L cefotaxim and 320 mg/L timentin. MMA contains MS basal salts without vitamins, 10 mM MES, 2 % sucrose and 200 µM acetosyringone (pH=5.6). IMM contains MS basal salts without vitamins, 500 mg/L glutamine, 100 mg/L casein hydrolysate, 1.95 g/L MES, 10 g/L glucose, 40 g/L maltose, 2mg/L picloram, 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 200 µM acetosyringone and 100 mg/L ascorbic acid. During co-cultivation period, MMD which is supplemented with 200 µM acetosyringone and 100 mg/L ascorbic acid and CCM were used. CCM medium was same as IMM medium but solidified with 2 mg/L phytagel. For the induction of transformed tissues, MS_2 and INM which was same as IMM without acetosyringone or glucose but supplemented with 0.5 mg/L 2,4-D, 160 mg/L timentin and 2 g/L phytagel. All of the vitamins, antibiotics and plant growth regulator were sterilized by using 0.2 µm pore sized filters and added freshly to sterile medium. The compositions and usages of transformation media are given in Table 2.2.

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Table 2.1. The compositions and usages of plant tissue culture media

Medium	Composition	Usage
MS_2	MS basal salts, 3 % sucrose ,2 mg / L 2,4-D , 0.28 % phytagel pH=5.8	Callus induction and maintenance
MS/2	½ MS basal salts , 3 % sucrose , 0.28 % Phytagel pH=5.8	Regeneration of wheat embryogenic calli
R_1	MS basal salts including Gamborg's B5 vitamins, 30 g/L maltose, 1 mg/L BA, 0.2 mg/L IAA, 2.8 g/L Phytagel pH=5.8	Regeneration of wheat embryogenic calli
R_2	MS basal salts including Gamborg's B5 vitamins, 30 g/L maltose, 1 mg/L Zeatin Riboside, 0.1 mg/L 2,4-D, 2.8 g/L phytagel pH=5.8	
I_1	MS basal salts including Gamborg's B5 vitamins, 30 g/L maltose, 2 mg/L 2,4-D, 2.8 g/L Phytagel pH5.8	Callus induction and maintenance
I_2	MS basal salts including Gamborg's B5 vitamins, 30 g/L maltose,2 mg/L Picloram, 2.8 g/L Phytagel pH=5.8	

Table 2.2. Medium compositions and usages for transformation study.

Medium	Composition	Usages
IMM	MS basal salts, 500 mg/L glutamine, 100 mg/L casein hydrolysate, 1.95 g/L MES, 10 g/L glucose, 40 g/L maltose, 2.2 mg/L picloram, 2 mg/L 2,4-D, 200 µM acetosyringone, 100 mg/L ascorbic acid pH=5.8	Bacterial inoculation of explants, elimination of *Agrobacterium* when supplemented with Cefotaxim (500 mg/L) and Timentin (160 mg/L)
MMA	MS basal salts excluding vitamins, 10 mM MES, 2 % sucrose pH=5.8	
CCM	Same as IMM medium, but solidified with 2 g/L Phytagel	Co-cultivation of wheat calli
MMD	MS basal salts, 10 mM MES, 3 % sucrose, 1 mg/L 2,4-D, 200 µM Acetosyringone, 100 mg/L Ascorbic acid, 0.28 % Phytagel pH=5.6	
INM	Same as IMM medium, but no acetosyringone or glucose and 0.5 mg/L 2,4-D.Add 160 mg/L Timentin and 2 g/L Phytagel pH=5.8	Induction of transformed calli

2.1.4. Bacterial Strains and plasmids

In transformation studies, *Agrobacterium tumefaciens* strain AGL I harboring pAL 154 and pAL 156 was used. AGL I is resistant to carbenicillin and the selectable marker for pAL 156 is *nptI* .pAL 156 includes *GUS* and *bar* marker genes. The map of the plasmids is given in Appendix C. The *Agrobacterium* strain including the plasmids was donated by Matthew D.Perry and Wendy Harwood from John Innes Centre. The transfer agreement is given in Appendix D

2.1.5. Bacterial Culture Media

YEB medium which contains nutrient broth, yeast extract, sucrose and magnesium sulphate and MG/L medium (Tingay *et al.* 1997) were used for the growth of AGL I. (Appendix E).The media were supplemented with kanamycin (100 mg/L) and carbenicillin (200 mg/L). For the large scale growth of AGL I, YEB+MES medium containing kanamycin (100 mg/L) and carbenicillin (200 mg/L), and 20 µM acetosyringone was used for the induction of *vir* genes. (Appendix E).

2.2. Methods

2.2.1. Tissue Culture Studies in Wheat

The effects of vernalization time and callus age on regeneration potential were examined.

2.2.1.1. Growth of the Plant

The explants were obtained from wheat plants which were grown in greenhouse. Kızıltan-91 and Bezostaja-01 seeds were sterilized with sodium hypochlorite. The seeds were sown in soil containing pots. Greenhouse conditions were set to 25±3 ° C and 16/8 h photoperiod. One week old wheat plants were transferred to cold room. They were exposed to 4° C for 1,2,3,4, and 5 weeks, respectively. Vernalization room conditions were set to 16/8 h photoperiod. After the cold treatment, plants were placed into the greenhouse. The wheat plants were fertilized in two weeks interval.

2.2.1.2. Isolation of Immature Inflorescence

In order to obtain immature inflorescence in the desired stage, tillers containing immature inflorescence were collected prior to the emergence of flag leaf and this period correspond to 50-60 days for Kızıltan-91 and 80-90 days for Bezostaja-01. Tillers were harvested when inflorescence were generally between 0.5-1.0 cm in length. Surface sterilization was performed by sequentially washing the tillers in 70 % ethanol for 30 seconds and then 20 % sodium hypochlorite for 25 minutes, followed by three rinses in sterile distilled water. All procedure that followed by sodium hypochlorite treatment were conducted under aseptic conditions.

The immature inflorescences were isolated from these tillers under stereomicroscope. Isolated inflorescences were cut into approximately 1mm sections (Figure 2.1).These pieces were plated on callus induction media (MS_2, I_1 and I_2). About 30 pieces were put onto each plate. After sealing, they were incubated in the tissue culture room at 25 ° C under dark conditions.

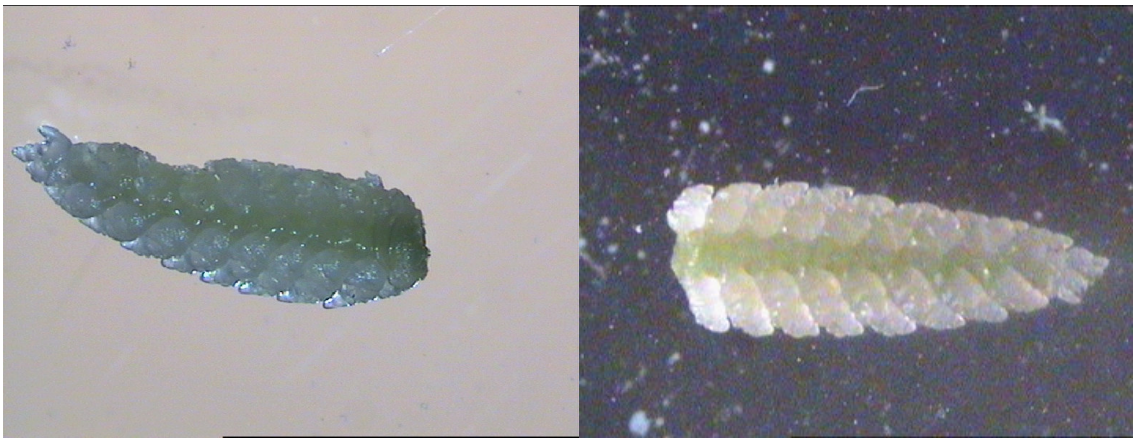

Figure 2.1. Appearance of immature inflorescences.

2.2.1.3. Determination of Vernalization Time on Inflorescence formation

In order to determine vernalization time, tillers containing immature inflorescences that belong to different vernalization period were harvested at the same time. After harvesting, the total tillers were scored and inflorescences were isolated under stereomicroscope. For determination of percentage of inflorescence formation, inflorescence formed tillers were counted.

2.2.1.4. Induction and Maintenance of Callus Cultures

For Kızıltan, the induction and maintenance of callus cultures were conducted on MS_2 medium. However, I_1 and I_2 medium were used for callus induction and maintenance for Bezostaja. These calli were subcultured at 4 weeks intervals, since the nutrient and plant growth regulator concentration decreases by time. As a result of nutrient limitation, the calli get into stress.

2.2.1.5. Determination of Callus Growth Rate

For the determination of callus growth rate, calli were placed onto a filter paper and weight of calli was measured under aseptic conditions. After the measurement, calli were placed onto used medium. This measurement was repeated every week for 5 weeks.

2.2.1.6. Determination of Effect of Callus Age on Regeneration Potential

Six weeks old calli were cut into two pieces. One piece was placed onto callus maintenance medium and kept at dark as previously while other piece was placed onto regeneration medium and transferred to light conditions. After 4 weeks in regeneration medium, regenerated plantlets were transferred into jars. This procedure was repeated 4 weeks interval up to 15 weeks. The same procedure was repeated for 9 weeks old calli (after 3 three weeks) and 12 weeks old calli which

kept in callus maintenance medium. At the 15th week, all of the calli which kept in callus maintenance medium were transferred onto regeneration medium.

2.2.1.7. Regeneration of Wheat Calli via Somatic Embryogenesis

For shoot and root initiation, calli were transferred into regeneration medium after dark incubation period. Kızıltan calli were placed onto regeneration medium in which strength of basal salts were reduced to half and 2, 4-D was excluded. Bezostaja calli were put onto R_1 medium including BA (1 mg/L), IAA (0.2 mg/L) and R_2 medium including Zeatin Riboside (1 mg/L) and 2,4-D (0.1 mg/L) for regeneration.

Root and shoot formation were observed accordingly. Data were collected after 4, 8, 12 and 15 weeks as the number of shoot regenerating explants over total explants (Figure 2.2).

Four weeks old plantlets obtained from regeneration medium were transferred into jars containing same regeneration medium. Two weeks after the transfer, jars containing plantlets were transferred into vernalization room. The plantlets were exposed to 4 ° C for 4 weeks. After the vernalization period, they were transplanted to soil. The soil was previously autoclaved and cooled in order to prevent any contamination and weed formation.

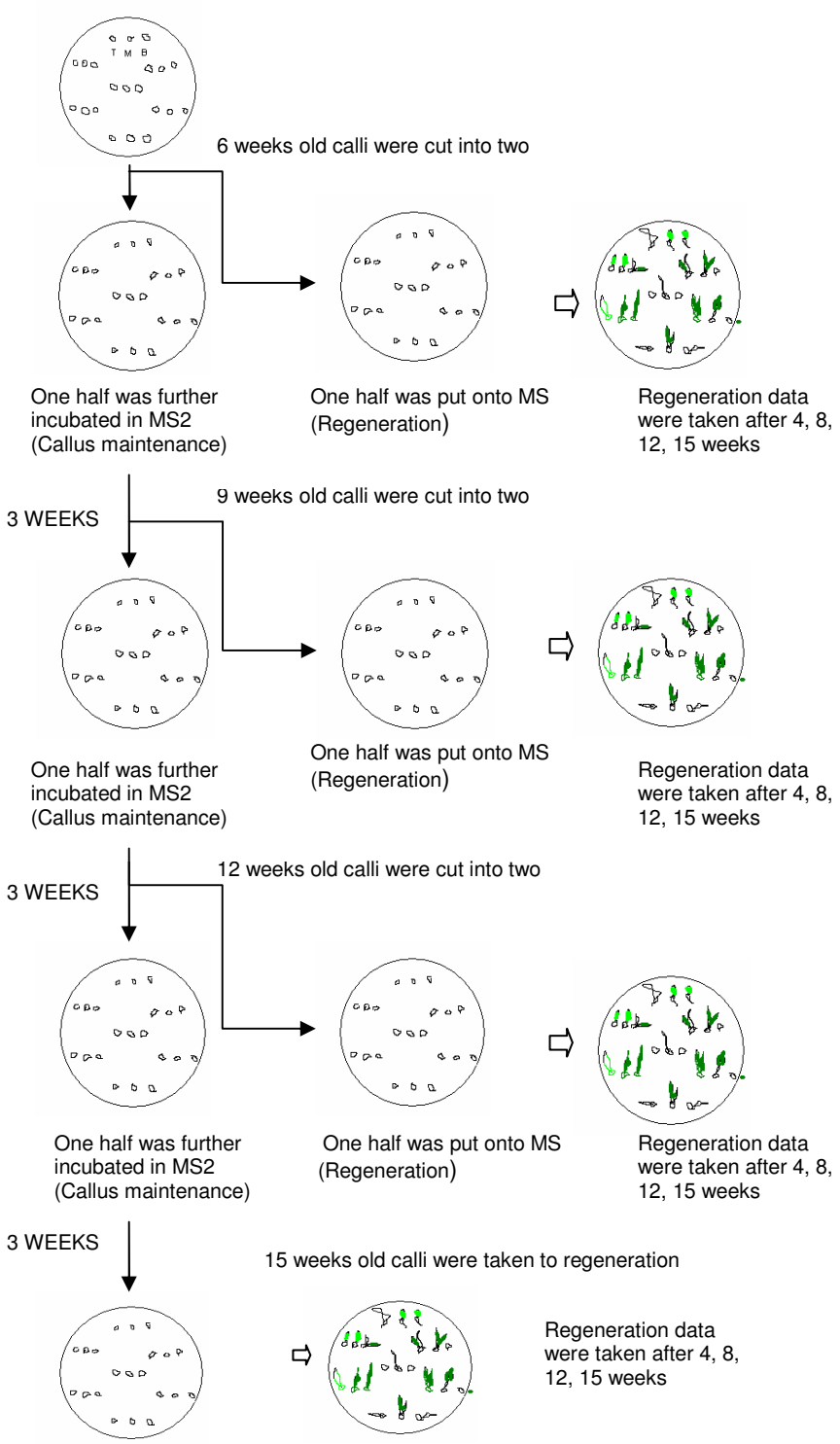

Figure 2.2.Schematic representation of experimental design for the determination of effect of dark incubation period on regeneration success.

For acclimatization, pots containing plantlets were covered with plastic bags in order 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 were completely removed.

Plants were watered regularly. They were fertilized in two weeks intervals and they became mature plants approximately 3 months after the transfer. When the plant became yellow and all seeds were mature, spikes were collected. The numbers of tillers and the number of spikes for each plant, the number of seeds and the weight of each seed were recorded.

2.2.2. *Agrobacterium*-Mediated Transformation of Wheat Inflorescence

Bezostaja and Kızıltan inflorescence based calli were transformed with *Agrobacterium tumefaciens* AGL I. Immature inflorescence were collected, isolated and placed onto callus induction medium. The inflorescences were kept at callus induction medium under dark conditions at 25 ° C .Transformation studies were performed by using calli at different ages.

2.2.2.1. Preparation of *Agrobacterium tumefaciens* AGL I cells

Agrobacterium tumefaciens strain AGL I were grown in liquid YEB and MG/L (Tingay *et al.*, 1997) medium supplemented with kanamycin (100 mg/L) and carbenicillin (200 mg/L). Single colonies were selected from YEB+agar culture and inoculated into 5 ml YEB cultures prior to transformation studies. Liquid bacterial cultures were incubated at 28 ° C at 180 rpm. There were two utilized protocol for *Agrobacterium* transformation of Bezostaja and Kızıltan inflorescence based calli. First protocol was adopted from Mahmoudian *et al.* (2002). Second protocol was adopted from Wu *et al.* (2003). Both of the protocols have similar stages and they were as follows:

i. induction of *Agrobacterium vir* genes,
ii. inoculation of wheat calli with *Agrobacterium*,
iii. co-cultivation of the bacteria and plant
iv. elimination of *Agrobacterium*.

Although they share similar transformation stages, they differ in the types of medium used.

2.2.2.2. Transformation Procedure

In first transformation protocol, bacteria were growth in YEB+MES medium which is supplemented with kanamycin (100 mg/L), carbenicillin (200 mg/L) and 20 µM acetosyringone as the phenolic compound. Since *Agrobacterium tumefaciens* cannot infect wheat in nature, the bacteria should be induced prior to transformation studies. According to the method developed by Kapila and co-workers (1997), in which bacteria are in log phase, they were inoculated into YEB+MES medium supplemented with kanamycin (100 mg/L), carbenicillin (200 mg/L) and 20 µM acetosyringone.

In second procedure adopted from Wu *et al.* (2003) bacteria *vir* genes grown in MG/L (Tingay *et al.* (1998) cannot be induce during growth, since this medium did not contain any phenolic compounds. The growth of the bacteria was monitored by OD measurement at 600 nm with Shimadzu UV visible spectrophotometer 1240.

When the optical density of the bacterial culture was reached to about 0.8-1.2, the bacteria were collected by centrifugation at 1500 g for 15 minutes. Then for actual activation of the bacterial *vir* genes, cells were resuspend in MMA (Mahmoudian *et al.*, 2002) or IMM (Wu *et al.,* 2003) media which contains 200 µM acetosyringone until optical density becomes 2.0-2.4. The bacteria were incubated at MMA or IMM media at 24 ° C for 60-80 minutes in dark conditions for the induction of *vir* genes (Table 2.2).

The bacteria become ready to infect plant cells during incubation period in MMA or IMM media. After the incubation period, the wheat calli were placed in bacterial suspension for 1 hour. Inoculation period was performed at -600 mmHg by using vacuum infiltration equipment with occasional shaking (Figure 2.3). Inoculum was then pipetted out and infected explants were blotted on sterile filter paper and then plated onto co-cultivation medium (MMD or CCM). Co-cultivation was carried out in the dark at 25 ° C for 3-4 days.

Figure 2.3. Vacuum infiltration equipment.

After co-cultivation period, calli were washed with MMA or IMM media supplemented with 500 mg/L cefotaxim and 320 mg/L timentin for 60 minutes. Then explants were blotted and plated onto MS_2 or INM media supplemented with 160 mg/L timentin and 1 mg/L PPT for 3-4 days in order to allow for recovery of transformed tissues.

Determination of lethal dose for regeneration

For the determination of the effects of PPT and timentin on regeneration, an experiment was designed. Six weeks old calli were put into regeneration medium including 0, 1, 2, 3, and 4 mg/L PPT and 0, 160, 200, and 300 mg/L timentin. In order to determine effects of PPT and Timentin on root formation, regenerated plantlets were put into jars containing 160 mg/L Timentin and 0, 1, 2, 4, 6, and 10 mg/L PPT. We also investigated the effects of PPT and timentin on callus growth. For this reason, 5 weeks old calli were put into callus induction medium containing 0, 3, 6, and 10 mg/L PPT and 160 mg/L timentin. After 5 weeks dark incubation period, we recorded percent change of callus weight.

2.2.2.3. Histochemical GUS assay

Seven days after transformation, histochemical GUS assay was performed according to the method of Jefferson (1987), in order to observe transient gene expression. For GUS assay, explants were put into 1 mM chromogenic substrate 5-bromo-4-chloro-3-indoyl-β-D-glucuronic acid (X-Gluc) containing GUS substrate solution (Appendix F) in falcon tubes and they were vacuum infiltrate at -600 mmHg for 1-2 minutes for better absorption of the substrate solution by the plant tissue. Then the explants were incubated overnight at 37 ° C and at 25 ° C for a further 24 hours in dark conditions. At the end of the incubation period, the explants were examined under stereomicroscope and scored for the number of explants producing blue spots per treatment as well as the number of spots per explants. For fixation of the color, the calli was stored in GUS fixative solution (Appendix F).

2.2.3. Statistical Analyses

All of the statistical analyses were carried out by using Minitab Statistical Software. Analysis of variance (ANOVA) was used to investigate the relationship between a response variable and one or more independent variables.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Determination of *vernalization* time on Inflorescence formation

In this study, two winter wheat cultivars were used (Kızıltan-91 and Bezostaja-01). In order to find optimum vernalization time for two cultivars, they were subjected to +4 ° C for different periods. This vernalization temperature was supported by several authors (Chujo, 1966; Havely, 1985; Petr, 1991). In these studies, the vernalization temperature has been suggested to be with in 2-6 ° C temperature range. In our study, for the determination of optimum vernalization time, wheat plants, are subjected to cold and grown in greenhouse, were collected when they were about 2 months old.

All the wheat plants which were subjected to cold with different incubation periods were collected at the same time. To find optimum vernalization time, inflorescences were isolated from these plants and data were recorded as the number of inflorescence formed tillers per total tillers (Figure 3.1).

When the inflorescence formations of Kızıltan cultivar were compared, there were differences between 2 weeks and 3 weeks cold treated plants (p: 0.011). However, there were no differences between 3 weeks, 4 weeks, and 5 weeks cold treatment (p: 0.226 for 3-4 weeks, p: 0.615 for 3-5 weeks, and p: 0.358 for 4-5 weeks cold treatment) in terms of inflorescences formation. Also figure 3.1 shows that percentage of inflorescence formation of Bezostaja cultivar. Statistical studies showed that in Bezostaja, there were significant differences between 3 weeks and

4 weeks cold treated plants, when the inflorescence formation results were compared (p: 0.001 for 3-4 weeks cold treated plants and p:0.000 for 3-5 weeks cold treatment plants) and also there were difference between 4 weeks and 5 weeks vernalised plants (p=0.077).

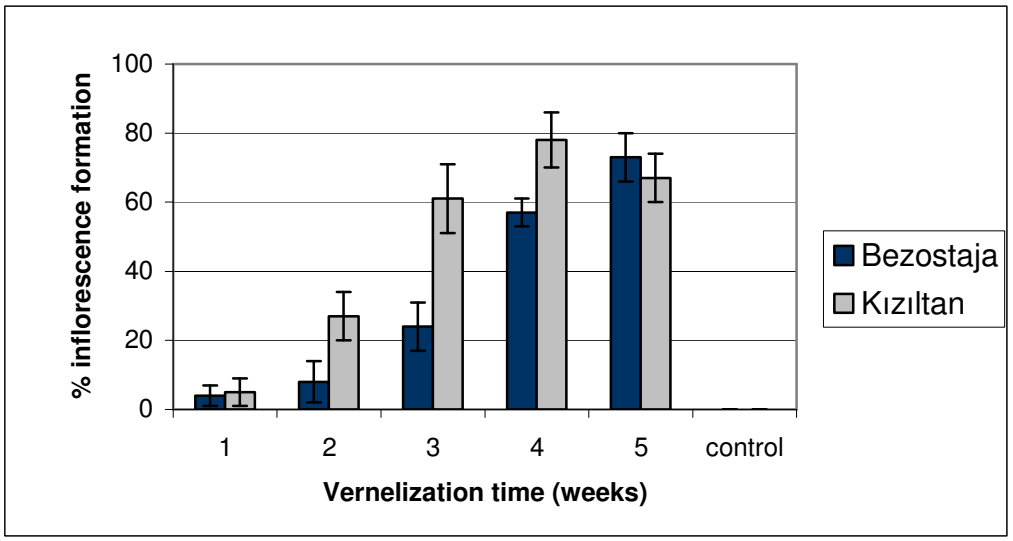

Figure 3.1.Effect of vernalization time on inflorescence formation. Control represents % inflorescence formation in non-vernalised plants.

Our results are comparable with others. Wang (1995), Robertson (1996), and Streck (2003) showed that vernalization time might vary by genotype, ranged from 21 to 52 days.

As a consequence of determination of inflorescence formation studies, only 3, 4, and 5 weeks vernalised plants were used for further studies.

During vernalization time determination studies, the effects of vernalization on flowering were also recorded. These studies were correlated with previous studies performed with different authors and showed that wheat plants respond to vernalization by decreasing the time for flowering (Slafer and Rowson, 1994; Cao and Moss, 1997; Wang and Engel, 1998). Figure 3.2 shows different bread wheat plants subjected to cold for different durations. Bezostaja cultivar vernalised for 2 and 3 weeks did not produce any spike.

Figure 3.2. The appearance of bread wheat plants with different vernalization time. Photograph was taken after 15 weeks in greenhouse.

3.2. Regeneration studies

In this section, callus induction and the effect of callus age on regeneration capacity together with the effect of vernalization time and regeneration time on regeneration capacity have been shown.

3.2.1. Callus induction

Immature inflorescences of Kızıltan-91 and Bezostaja-01 were used as the explant sources. Tillers containing inflorescences were harvested when inflorescences were generally between 0.5-1.0 cm in length. This stage was found to respond best in callus formation and was shown to be highly responsive to regeneration by several authors (Sharma *et al.*, 1995; Caswell *et al.*, 2000; Benkirane *et al.*, 2000).

In Kızıltan cultivar, callus induction was initiated from about 1mm immature inflorescences fragments by using MS_2 medium including 2,4-D (2mg/L). Somatic embryos were successfully produced and regeneration took place by using half strength MS.

In Bezostaja cultivar, callus induction was initiated from about 1 mm immature inflorescence fragments by using MS_2 including 2 mg/L 2,4-D, I_1 including 2 mg/L 2,4-D and I_2 including 2 mg/L picloram. In preliminary studies, MS_2 medium was used for callus induction. Although callus induction was successfully performed, somatic embryos were not established in MS_2 medium after 10 trials. Therefore, the other medium namely I_1 and I_2 were also used. Compositions of these media are given Table 2.1. As a result of sets of experiments, I_2 medium was found to be more responsive for callus induction. Our results seem to be consistent with the literature. He and Lazzeri (2001) reported that regeneration frequencies from inflorescence cultures induced on picloram showed more than a two-fold increase over the values for cultures induced on 2,4-D.

Callus growth curves were obtained by weighing immature inflorescences in one week intervals for 5 weeks. Figure 3.3 shows callus weight increasing per plate.

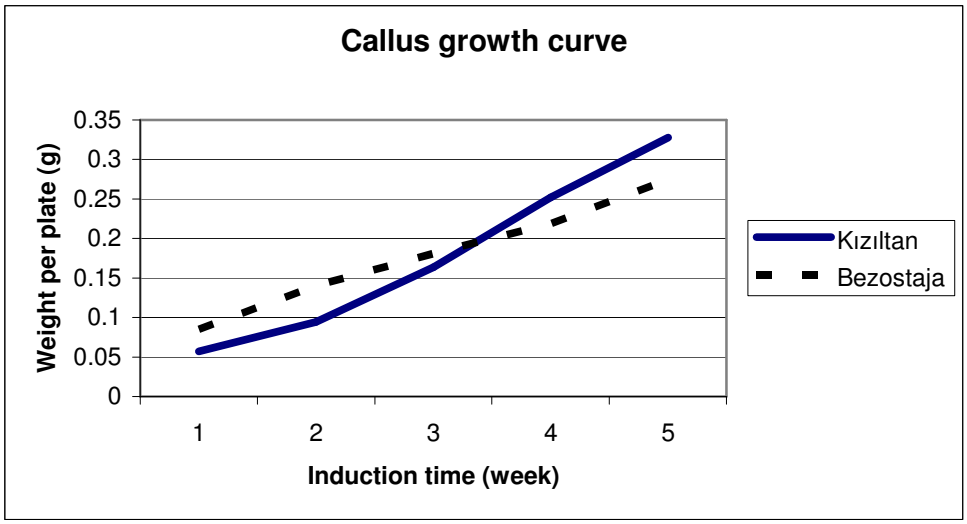

Figure 3.3. Callus growth curve for Kızıltan and Bezostaja.

In this study, callus formation became visible after 1-2 weeks dark incubation period for both cultivars. Kızıltan's callus formation took place faster than Bezostaja. In 1982, Ozias-Akins and Vasil reported that the young inflorescence segments callused on 2 mg/L 2,4-D only after an extended lag period. They also indicated that within a few days, the rachis and bases of the glumes became bright green but signs of callusing were not evident for two weeks. Therefore, our results seem to be consistent with the literature. Stages of callus formation are given in Figure 3.4 for two cultivars.

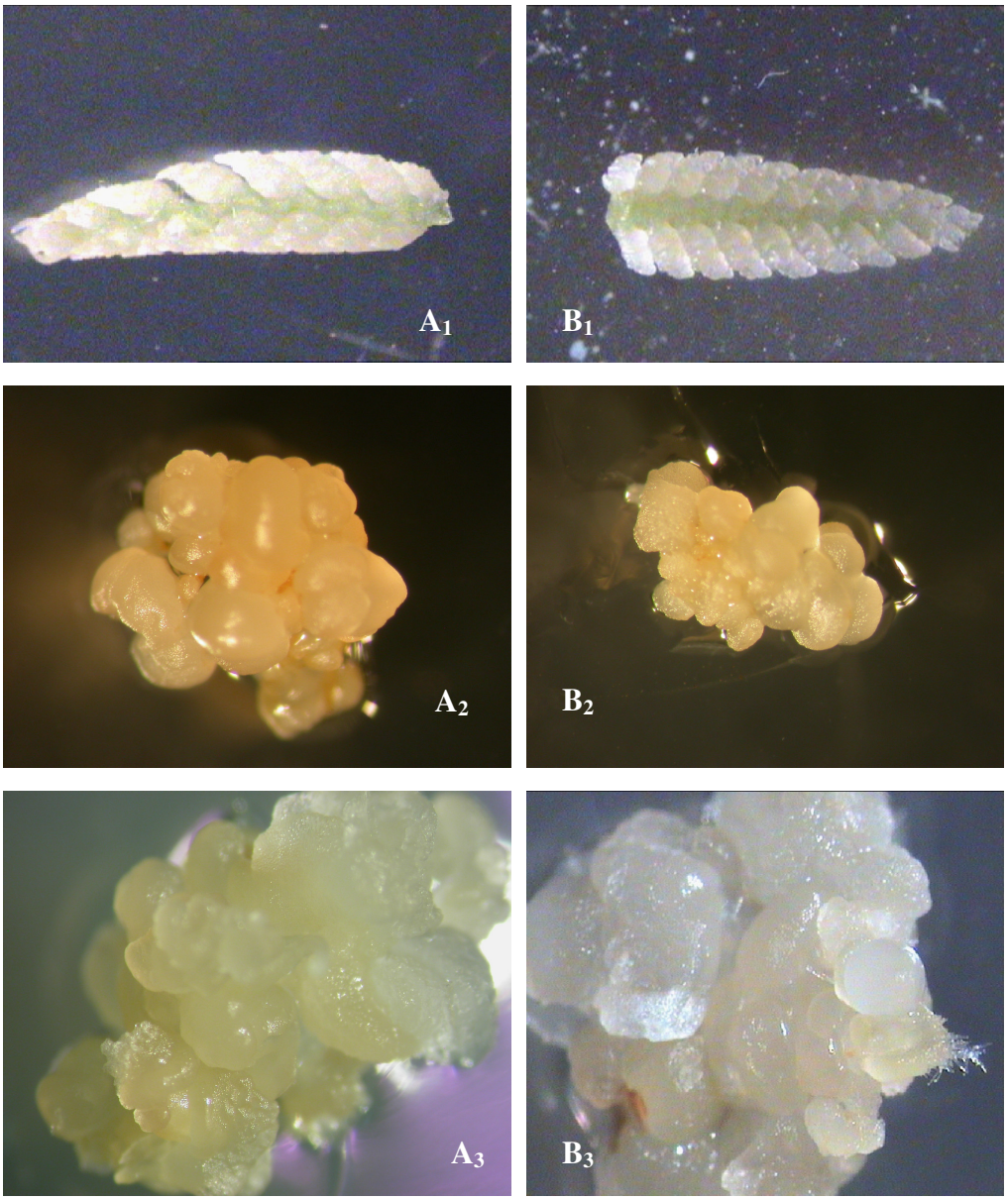

Figure 3.4. Callus development from Kızıltan (A) and Bezostaja (B). A_1-B_1:Freshly isolated immature inflorescence. A_2-B_2: Callus formation after 2 weeks in callus induction medium. A_3-B_3: Four weeks old calli in callus induction medium.

All of the explants put onto callus induction medium not responded and some of them did not produce callus especially for very small inflorescences. The size of inflorescences depended on vernalization time. As we collected all the plants belong to different vernalization period at the same time, one and two weeks vernalised plants and 3 weeks vernalised plants were generally formed very small inflorescences for Kızıltan and Bezostaja, respectively.

3.2.2. Effect of callus age on different regeneration parameters depending on vernalization time

In this study, effects of callus age on embryogenic capacity, shoot development and seed characteristics were investigated.

3.2.2.1. Embryogenic capacity

In order to demonstrate the effect of callus age on embryogenic capacity, calli were incubated for 6 weeks, 9 weeks, 12 weeks and 15 weeks in callus induction medium in dark. For each callus age, there were 3 different types of calli which were different in terms of explants sources' vernalization time, namely 3 weeks, 4 weeks, and 5 weeks vernalised plants.

In previous studies for Bezostaja, regeneration medium which is free of growth regulators and contains MS salts in half strength was used. However, there were no somatic embryo development and no regeneration took place. We observed only root formation by using this medium. After 10 trials, we decided to use alternative regeneration media, namely R_1 (BA and IAA) and R_2 (Zeatin Riboside and 2,4-D). According to Vasil (1987) this root formation indicated that this callus was non-embryogenic because non-embryogenic calli can produce solely roots but no shoots (Figure 3.5).

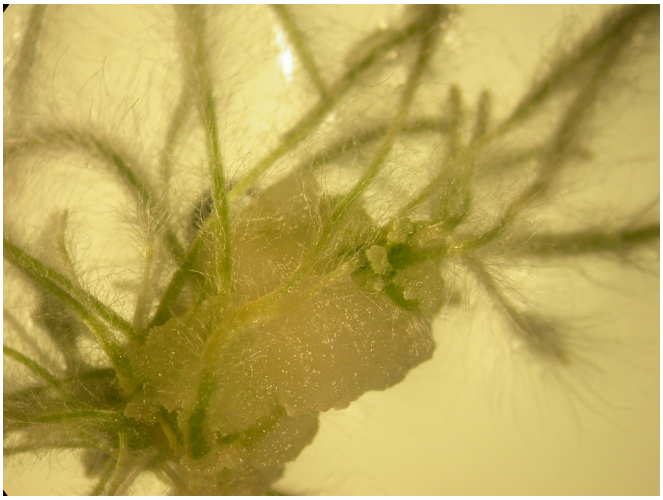

Figure 3.5. An inflorescence derived non-regenerating callus.

At the end of the dark incubation period, half of each callus was taken to light on regeneration media MS/2 and R_1. Germination of somatic embryos are usually induced on hormone-free culture medium or medium that contains low levels of an auxin or low levels of both an auxin and a cytokinin (Carman, 1995). The germination of somatic embryos were observed after their transfer to light as illustrated in Figure 3.6.

Ozias-Akins and Vasil have observed formation of discreet nodules prior to germination of the somatic embryos in immature embryo derived calli (Ozias Akins and Vasil, 1982). Later on, trichomes formed on the smooth nodular areas and they differentiate a leafy structure by the formation of a notch on the surface of a nodule, and finally the embryo-like structures were observed. In Kızıltan callus, at the end of 6 weeks period the similar globular structures were observed. This structures can be interpreted as those “notches” described by Vasil or can be considered as globular stage which is the first sequential stage of embryo formation in the somatic embryogenesis process (Figure 3.6 and 3.7).

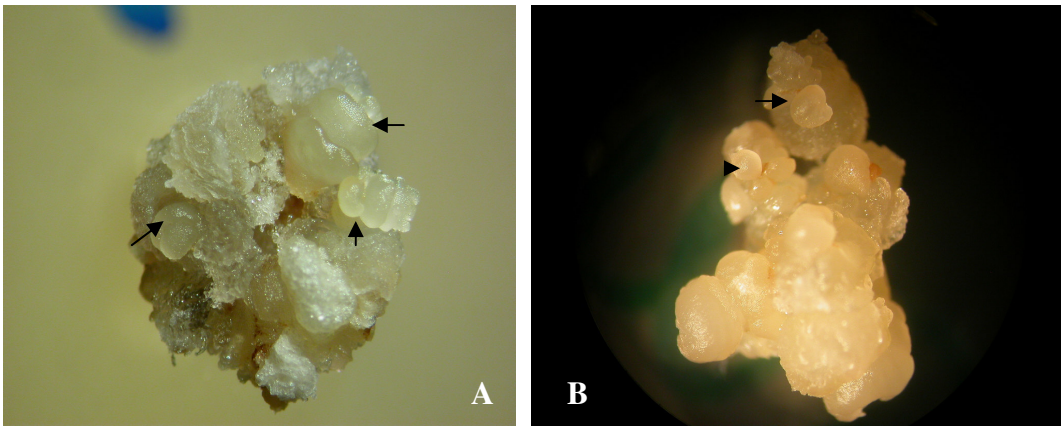

Figure 3.6. The germination of somatic embryos. **A.** Globular and heart shape structure of 30 days old immature inflorescence-derived callus (Bezostaja). **B.** The globular structure of 36 days old callus (Kızıltan).

Figure 3.7. The embryoid structure from one week old callus (Kızıltan).

In 7-10 days after their transferred to regeneration medium, the emergence of green shoots and roots were observed (Figure 3.8).

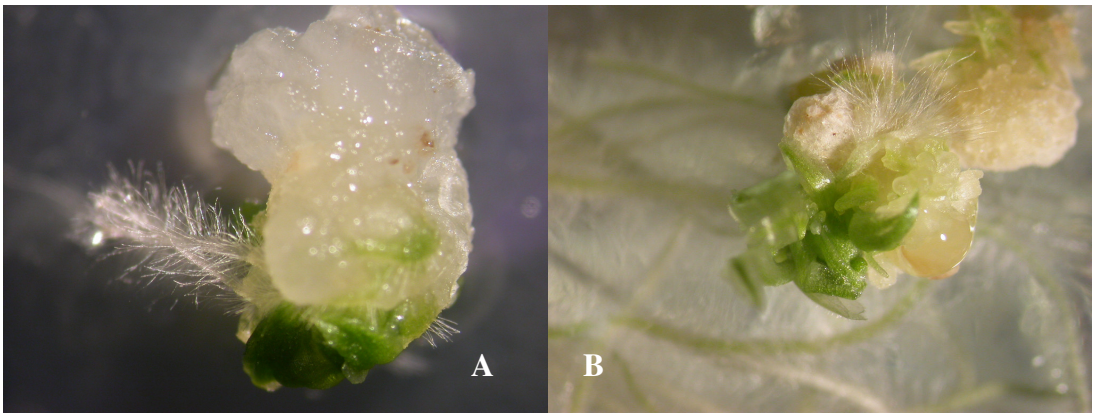

Figure 3.8. Regeneration from immature inflorescences derived calli.
A.10 days old calli in regeneration medium for Bezostaja. B. 7 days old calli in regeneration medium for Kızıltan.

The calli were incubated on regeneration medium for 4 weeks. At the end of four weeks, regenerated plantlets were transferred into jars containing regeneration medium and non-regenerated calli were placed onto fresh regeneration medium. This procedure was repeated at four weeks interval for 15 weeks. Data were collected as regenerating calli per total calli when the calli were subcultured onto fresh medium. Figure 3.9 shows regenerating calli ready to transfer to jars and transferred plantlets jars.

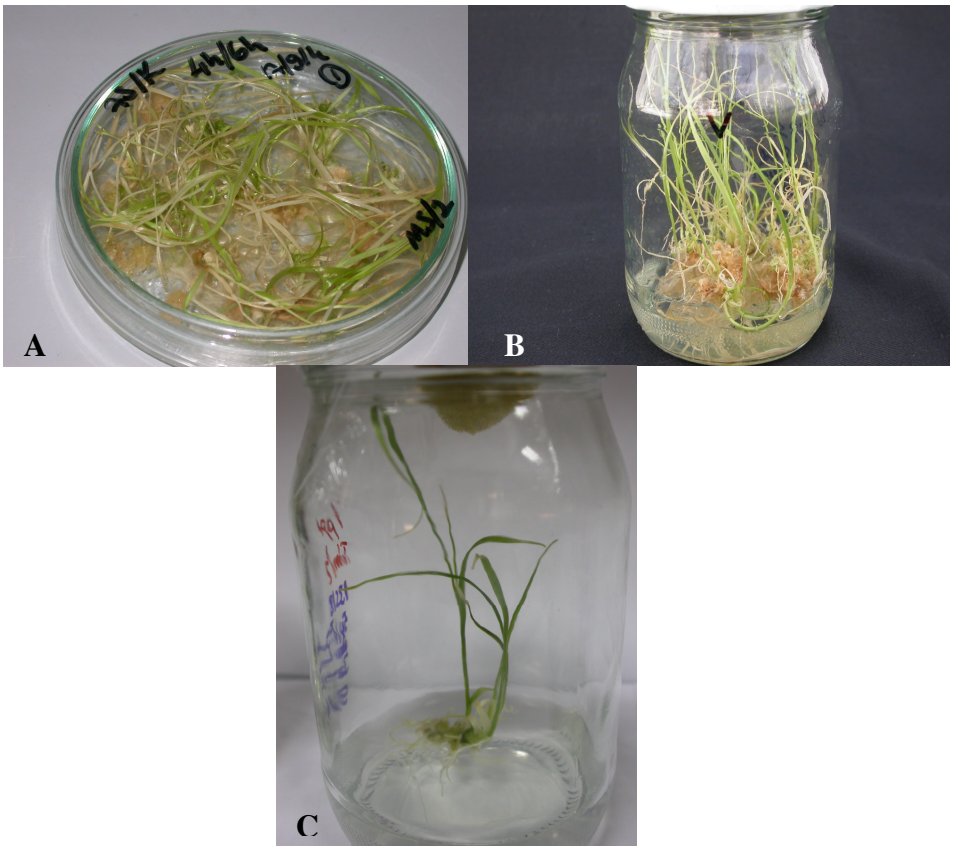

Figure 3.9. Renerating calli and transferred plantlets. A. Regenerating calli incubated in regeneration medium for four weeks (Kızıltan). B. Plantlets incubated in jar for four weeks (Kızıltan). C. Plantlets incubated in jar for 2 weeks (Bezostaja).

Two weeks after transfer, plantlets were moved to vernalization room for cold treatment at 4 ° C for 4 weeks. Some plantlets referred to as control plants were not subjected to cold after jar transfer. At the end of 4 weeks, they were transferred to greenhouse conditions for acclimatization. Figure 3.10 show control and vernalised plantlets for Kızıltan cultivar.

Figure 3.10. Four week vernalised plantlets after two weeks in regeneration medium (**A**) and after transferred to pots in greenhouse (**B**). **v**: 4 weeks vernalised plants. **c**: Control plants.

We observed that there were no visible differences between controls and vernalised plantlets in terms of morphology (Figure 3.10). Similarly, He and Lazzeri (2001) applied three vernalization procedure and they observed that regenerated plants were indistinguishable in terms of height, morphology and flowering time. Therefore, our results seem to be in accordance with literature.

Effect of regeneration time on regeneration frequency

This study was performed only using Kızıltan calli. As mentioned above, regeneration data were collected at 4 weeks interval for 15 weeks. Regeneration frequencies were increased by the increasing time. Figure 3.11 shows increased regeneration potential by the increasing time on regeneration medium at 6 weeks old calli belonging Kızıltan cultivar.

When we took into account calli, produced from explants subjected to 4 weeks vernalization, longer regeneration time enhances the shooting response. As it is

shown in Figure 3.11, percentage of shoot formation increased from 22.38 % to 69.6% by extending the incubation time from 4 weeks to 15 weeks. For the regeneration of lately developed somatic embryos, prolonged time in regeneration medium seems to be very important. It is important for transformation studies during which longer incubation periods for selection of transformants might be required either before or after taken to regeneration. The explants which pass through the transformation process are more delicate and may require more time for the development of somatic embryos. Thus, the explants should be kept in regeneration medium even longer than 8 weeks following transformation experiments.

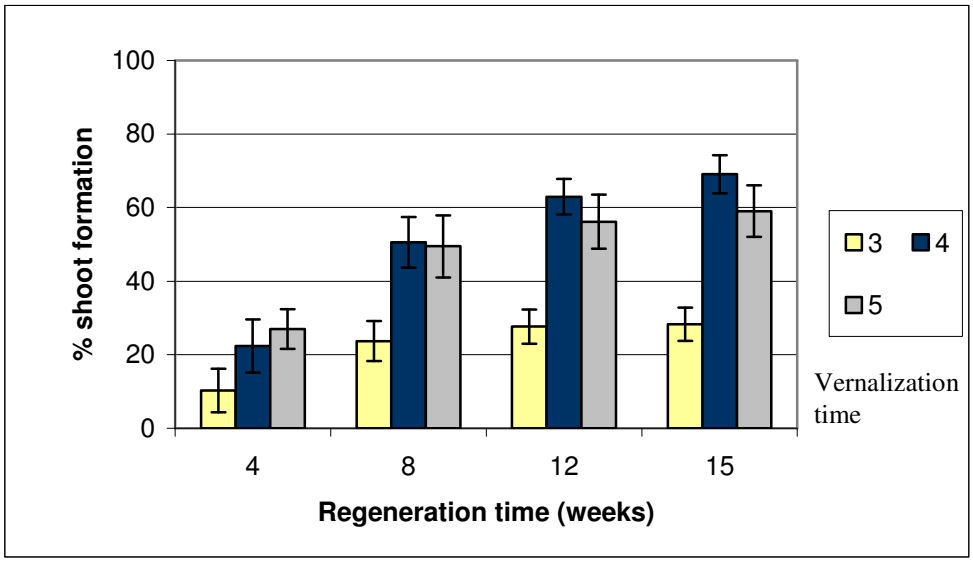

Figure 3.11. Increase in regeneration capacity at different vernalization periods by the time (Kızıltan).

The regeneration frequency of Kızıltan immature inflorescence tissue was very high when compared with other studies. In the report of Redway and co-workers

(1990), immature embryos were found to be more suitable for regeneration. However, they had transferred the calli to regeneration medium after 4 weeks of dark incubation period. In literature, for immature inflorescences the regeneration frequencies were found to be within the range of 28-100 % by Maddock *et al* (1983) and 45-80 % by He and Lazzeri (2001). For immature embryos, regeneration frequencies were found to be 12 % to 96% (Maddock *et al.*, 1983), 2% to 94% (Fennell *et al.*, 1996), and 60% to 100 % with overall mean 71.2% (Bomnineni and Jauhar, 1996). For mature embryos, the regeneration potential were found to range between 26.8 % to 97.8 % (Özgen *et al.*, 1996); with mean values of 39 % (Zale *et al.*, 2004), 70.4 % (Özgen *et al.*, 1996), 96.1 % (Özgen *et al.*, 1998). The advantages of using inflorescences over immature embryos are that they require less time for formation. Also explant isolation procedures are less time consuming. Immature inflorescences are primary explants, which are less likely than callus cultures to produce abnormal plants (Barcelo *et al.*, 1994). Usually, the cultivars used in biotechnology and tissue culture studies do not posses superior agronomic or quality traits (Zale *et al.*, 2004). Kızıltan is one of the good Turkish cultivars which is suitable for climatic conditions of Turkey. When these positive aspects are considered, Kızıltan immature inflorescence is suggested as a good candidate in transformation studies for improvement of Turkish wheat varieties.

For determining the effects of callus age on regeneration capacity, numbers of shoot regenerating calli were recorded at four weeks interval for 15 weeks. Cumulative data were collected at the end of 15 weeks of incubation period on regeneration medium for different callus age, that is, 6 weeks, 9 weeks, 12 weeks, and 15 weeks old calli (Table 3.1).

Table 3.1. Effect of callus age on regeneration capacity for Kızıltan (Data were collected at the end of 15 weeks of incubation on regeneration medium).

Callus Age	Vernalization Time	Number of callus	Number of shoot regenerating callus	Percentage of shoot induction
6 week	3 week	189	51	26.9
	4 week	264	174	65.9
	5 week	165	99	60
	Cumulative	**618**	**324**	**52.42**
9 week	3 week	188	57	30.3
	4 week	307	167	54.39
	5 week	187	94	50.26
	Cumulative	**682**	**318**	**46.62**
12 week	3 week	147	22	14.96
	4 week	277	94	33.93
	5 week	240	84	35
	Cumulative	**664**	**200**	**30.12**
15 week	3 week	196	24	12,24
	4 week	170	63	37.05
	5 week	134	49	36.56
	Cumulative	**500**	**136**	**27.2**

Since one of the characteristics of non-embryogenic calli is responding to regeneration only by rooting, rooting data were not used as an indicator of embryogenic capacity (Vasil, 1987; Felföldi, 1992).

When the shooting frequencies (number of shoot regenerating calli/ total number of calli) of calli in terms of callus age depending on vernalization time were compared, there were no significant changes between 6 weeks old calli and 9 weeks old calli obtained from explants subjected to cold for 3 weeks. However,

there were differences between 9 weeks old calli and 12 weeks old calli same as 12 weeks old calli and 15 weeks old calli. (Figure 3.12 p= 0.831 for 6-9 weeks, p=0.125 for 9-12 weeks, p=0.215 for 12-15 weeks).

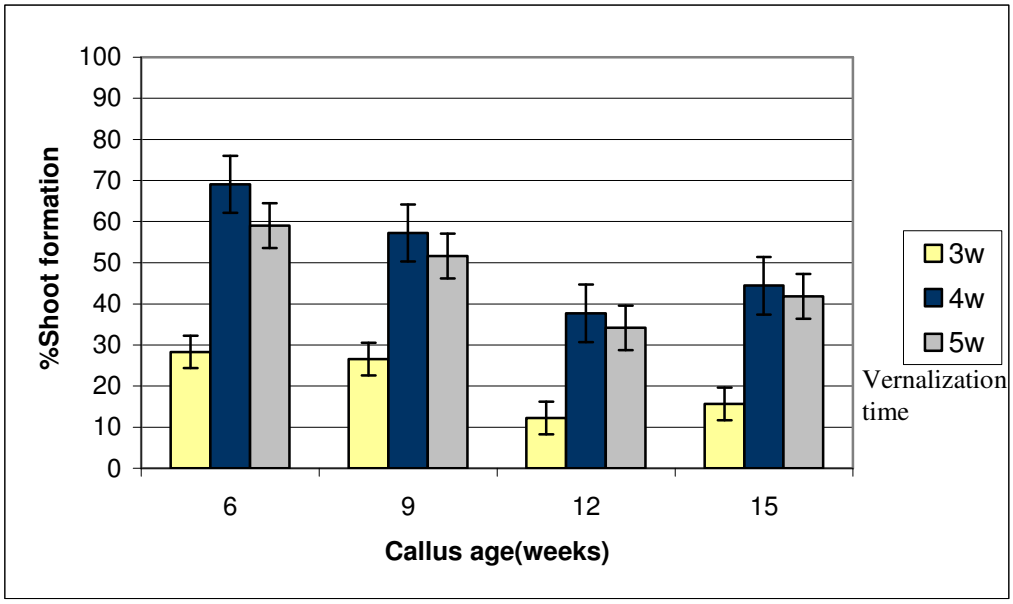

Figure 3.12. Effect of callus age and vernalization time on regeneration potential (Kızıltan).

Likewise, when we compared the shoot formation of calli obtained from 4 weeks vernalised plants, there were no differences between 6 weeks old calli and 9 weeks old calli. On the contrary, we observed significant difference between 9 weeks and 12 weeks old calli. In the same manner, 12 weeks and 15 weeks old calli have different regeneration capacity. (Figure 3.12 p=0.181 for 6-9 weeks, p=0.086 for 9-12 weeks and p=0.702 for 12-15 weeks).

In the same way, we observed no significant changes in terms of regeneration capacity in calli belong to 5 weeks vernalised plants. In contrast to calli, induced from 3 weeks and 4 weeks vernalised explants, there were no difference between 9 weeks old calli and 12 weeks old calli in terms of regeneration capacity same as 12 weeks and 15 weeks old calli ($p=0.607$ for 6-9 weeks, $p=0.021$ for 9-12 weeks, $p=0.152$ for 6-15 weeks).

When we analyzed the data in terms of vernalization time, calli produced from explants obtained from 4 weeks and 5 weeks vernalised plants were formed shoot significantly higher than 3 weeks vernalised ones at different callus ages. However, there were no differences between calli produced from explants obtained from 4 weeks and 5 weeks vernalised plants in terms of embryogenic capacity at different callus ages. In literature, there was no study regarding with the effect of vernalization time on regeneration capacity.

Vasil (1987) has indicated that many researchers described the formation of green, leafy structures prior to shoot development. However, from a developmental point of view leaves are formed only after the organization of a shoot meristem. According to Vasil, the so called green leaves reported earlier in immature embryo-derived calli were actually the enlarged scutella which became green and leafy in precociously germinating somatic embryos. For inflorescence-derived calli, similar situation might be relevant. Especially in 15 weeks dark treatments, instead of formation of leaves after 12 weeks in regeneration medium, some green regions were observed (Figure 3.13). However, those regions have not given rise to shoots and the shooting efficiency of prolonged dark incubation was found lower.

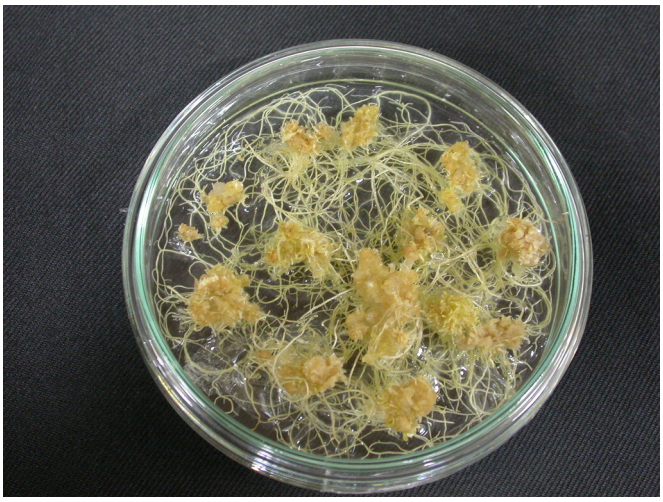

Figure 3.13. 15 weeks old calli at the end of 15 weeks incubation on regeneration medium.

Bezostaja

When compared to Kızıltan in terms of regeneration capacity, Bezostaja have lower regeneration capacity than Kızıltan at the end of 12 weeks incubation period. When we consider 4 weeks vernalised plants, regeneration capacity of Kızıltan has reached to 62.9 % at the end of 12 weeks with 6 weeks old calli. However, regeneration capacity of Bezostaja has only reached to 17.2 %. We obtained this regeneration frequency by modifying the composition of the medium especially changing the plant growth regulators. When we look at literature, Özgen and his colleagues (2001) reached to 85 % regeneration capacity by using mature embryos of Bezostaja.

Since three weeks vernalised plants were produced very small inflorescences not induced for callus formation, only four and five weeks vernalised explants were used for Bezostaja regeneration studies. When we compared Bezostaja and Kızıltan, we have lower data for Bezostaja than Kızıltan, as we had to try several

media for callus induction and regeneration in Bezostaja tissue culture studies. Table 3.2 shows regeneration data depending on vernalization time.

Table 3.2. Effect of callus age on regeneration capacity for Bezostaja.

Callus Age	**Vernalization Time**	**Number of callus**	**Number of shoot regenerating callus**	**Percentage of shoot induction**
6 weeks	4 week	151	26	17.2
	5 week	110	12	15.45
	Cumulative	**261**	**43**	**16.47**
9 weeks	4 week	107	12	11.21
	5 week	90	24	26.66
	Cumulative	**197**	**36**	**18.27**
12 weeks	4 week	111	12	10.8
	5 week	88	11	12.5
	Cumulative	**199**	**23**	**11.55**

When we analyzed the results, we observed that there were no differences between 6 weeks, 9 weeks, and 12 weeks old calli in terms of regeneration capacity in both calli obtained from 4 weeks vernalised explants and 5 weeks vernalised explants (Figure 3.14) (p=0.416 for 6, 9 and 12 weeks (4 weeks vernalization) and p=0.362 for 6, 9, and 12 weeks (5 weeks vernalization).

Data obtained from calli, produced by explants vernalised 4 weeks and 5 weeks, were compared, there were no changes in terms of embryogenic capacity at different callus age (p=0.18 for 6 weeks old calli, p=0.7 for 9 weeks old calli, p=0.49 for 12 weeks old calli). Data were collected from 6 weeks, 9 weeks, and 12 weeks old calli at the end of the 12 weeks in regeneration, since there were less data.

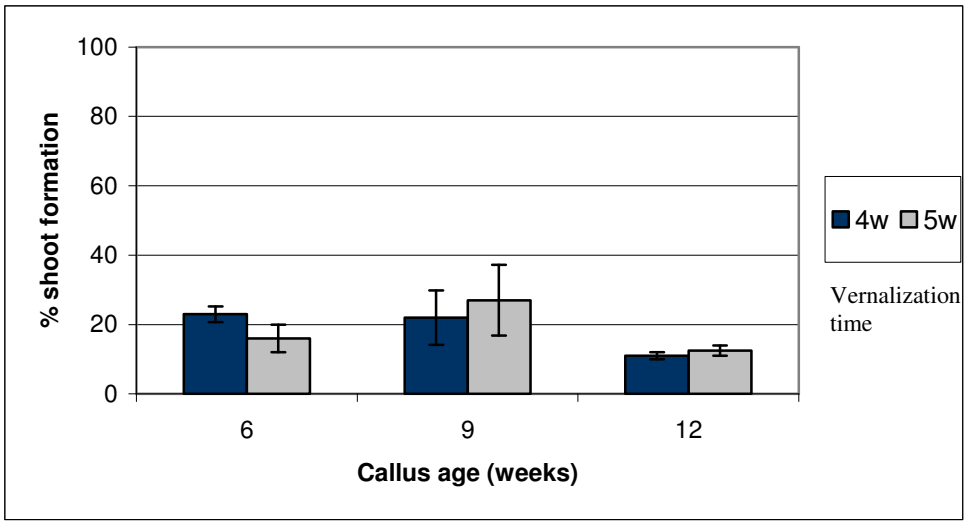

Figure 3.14. Effect of callus age on embryogenic capacity along with vernalization time (Bezostaja).

3.2.2.2. Shoot development

For shoot development and seed characteristics, only data obtained for Kızıltan will be given due to the incomplete studies on Bezostaja. Since all of the calli taken onto regeneration medium do not produce shoots with the same rate, the differences in shoot number might be different in explants. After their transfer to jars, plantlets were grown 2 weeks and they were transferred to vernalization room and they subjected to 4 ° C for 4 weeks. Some of the plantlets were not subjected to cold treatment after they are transferred to jars and they were called as control plants. We applied 4 ° C for 4 weeks, since we found that optimum vernalization time was 4 weeks as mentioned before. After vernalization, they were transferred to greenhouse and they were acclimatized to greenhouse conditions (Figure 3.15).

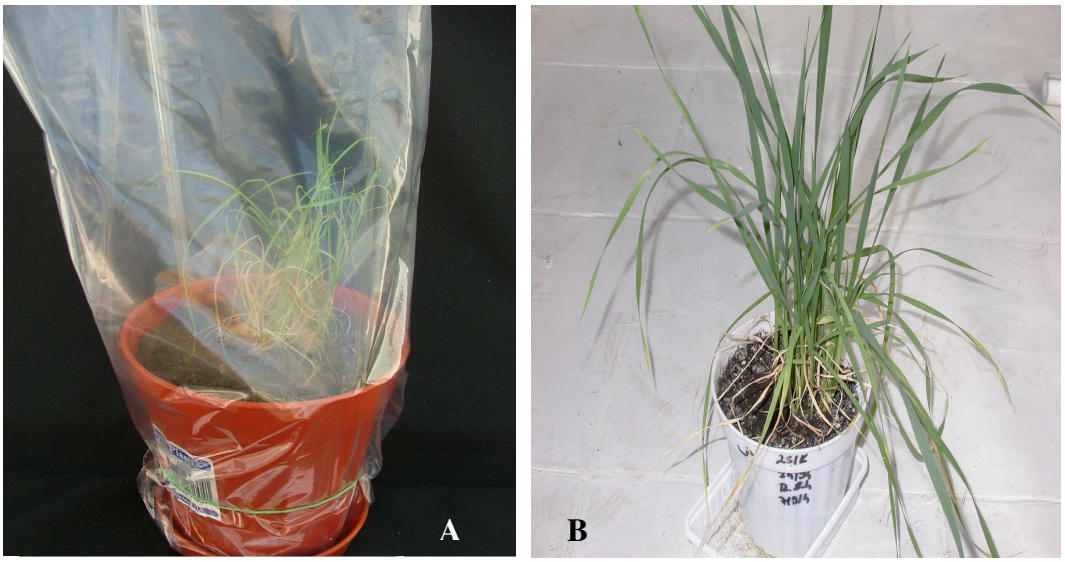

Figure 3.15. Acclimatization of plants (Kızıltan). A. Seven days after transfer to greenhouse B. Three weeks after transfer to greenhouse.

The tissue culture grown plants are more sensitive to environmental changes. Therefore, the temperature and relative humidity were very important during the acclimatization process. Although an extensive care was shown to tissue culture originated plants, some of them were lost during acclimatization. However, during the experiments, the mortality rate did not reach to a high percentage.

During soil transfer, data were recorded as the number of tillers per regenerated callus for both control plants and vernalised plants. Also total numbers of plants transplanted to soil were recorded (Table 3.3).

Table 3.3. Total number of plants transferred to soil.

CALLUS AGE	VERNALIZATION TIME	CONTROL	VERNALIZED
6 WEEKS	3W	5	9
	4W	56	74
	5W	12	20
	Total	**73**	**103**
9 WEEKS	3W	17	18
	4W	41	49
	5W	10	29
	Total	**68**	**94**
12 WEEKS	3W	4	6
	4W	20	25
	5W	9	15
	Total	**35**	**46**
15 WEEKS	3W	6	9
	4W	13	9
	5W	7	4
	Total	**26**	**23**
OVERALL	**TOTAL**	**202**	**266**

When control and vernalised plants were compared in terms of shoot development, there were difference between control and vernalised plants when the 6 weeks old calli obtained from 4 weeks vernalised explants were considered (p=0.119). In contrast, there were no difference between control and vernalised plants obtained from 9 and 12 weeks old calli (p=0.545 and p=0.267). Also, shoot development of control and vernalised plants were significantly different from each other, when 15 weeks old calli were considered (p=0.031). Figure 3.16 shows average number of tiller during soil transfer for control and vernalised plants. Results with standard errors are given in Appendix G).

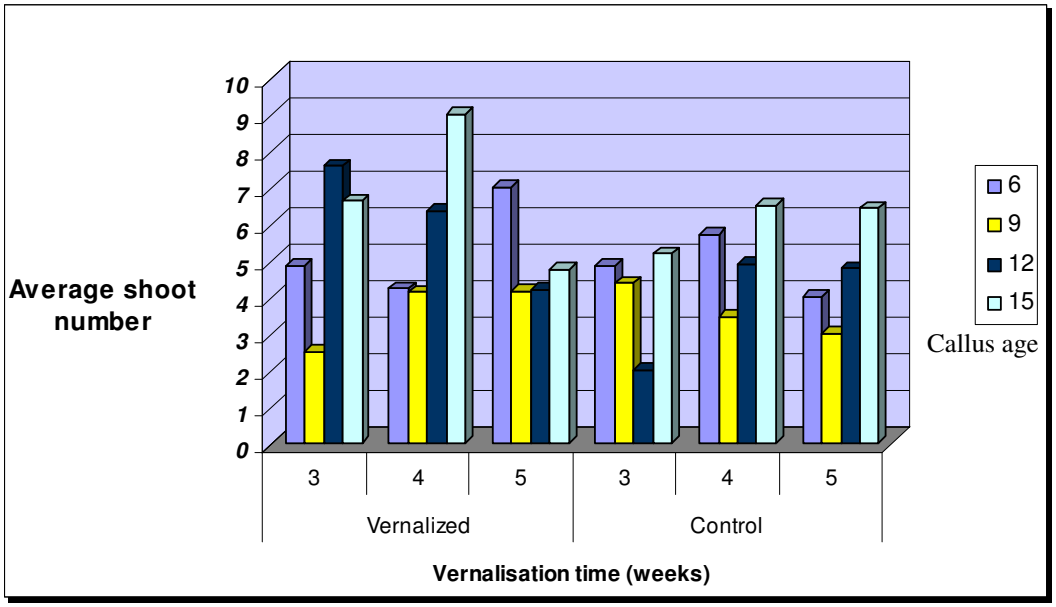

Figure 3.16. Average shoot number of Kızıltan during soil transfer (Control and vernalised plants).

When we consider callus age in terms of average shoot number during soil transfer, 15 weeks old calli were significantly different than the others (p=0.001 in

95 % confidence interval), if we take into account vernalised plantlets. However, there were no differences between callus ages in terms of average shoot number, when we consider the control plants (p=0.09 in 95 % confidence interval). In 2001, He and Lazzeri have reached to 6 average tillers by the help of BAP. However, we reached to 9 average tillers by using Kızıltan calli treated with 2,4-D. And also our results were consistent with Demirbaş (2004) who found that there were no differences between callus ages in terms of tillers.

Another comparison can be made on shoot development depending on vernalization time. Figure 3.16 also shows average shoot number belong to control plants during soil transfer obtained from different types of explants isolated from 3 weeks, 4 weeks and 5 weeks vernalised plants. We obtained the highest number of tillers in 15 weeks old calli as 5.17, 6.50 and 6.42 from 3 weeks, 4 weeks, and 5 weeks vernalised explants, respectively. When we analyze the data, there were no differences in terms of shoot development at different vernalization time.

We also compare the average shoot number of plantlets subjected to 4 weeks vernalization after jar transfer. There were no correlation between callus age and vernalization. Although statistical results show no difference between vernalization times at different callus age, we reached to maximum average tillers by using 15 weeks old calli, induced from 4 weeks vernalised explants, as 9. This result was higher than the He and Lazzeri (2001) result that they reached to 6 average tillers by the help of BAP.

3.2.2.3. Seed characteristics

Possession of high number of seeds with superior quality is one of the most important characteristics of a regenerated plant. The appearance of spike formations in control and vernalised plants, subjected to cold for 4 weeks after jar transfer, were demonstrated in Figure 3.17. As He and Lazzeri (2001) stated that

there were no difference between control and vernalised plants in terms of morphology.

Figure 3.17. The appearance of spike formation in control and vernalised Kızıltan wheat.

In order to monitor this traits, the number of spikes produced by a single plant and number of seeds together with their total weights were recorded. The data for total number of spikes and seeds belong to control regenerated and vernalised plants, subjected to cold after jar transfer, are given in Table3.4.

Table 3.4. Total number of spike and seed (Kızıltan).

Callus Age	Vernalization Time Plants (control-vernalised)	Total number of spikes		Total number of seeds	
		Control	Vernalised	Control	Vernalised
6 WEEKS	3W (0 plant/9 plants)	0	34	0	317
	4W (56 plants/72 plants)	273	269	908	405
	5W (8 plants/20 plants)	28	197	231	339
9 WEEKS	3W (17 plants/13 plants)	55	30	275	280
	4W (41 plants/49 plants)	138	186	610	714
	5W (10 plants/29 plants)	19	89	141	535
12 WEEKS	3W (4 plants/3 plants)	8	16	1	44
	4W (20 plants/25 plants)	89	117	498	473
	5W (9 plants/15 plants)	33	40	228	187
15 WEEKS	3W (6 plants/9 plants)	35	53	94	251
	4W (13 plants/9 plants)	84	66	398	200
	5W (7 plants/4 plants)	41	20	134	60

Most of the spikes were phenotypically normal and they contained seeds (Figure 3.18). However, some of the seeds were not well-developed. These undeveloped seeds may be due to unsatisfactory conditions in greenhouse. Also, there were many abnormal spikes (Figure 3.19). Durusu (2001) and Demirbaş (2004) stated that some of the seed obtained from tissue culture were small and wrinkled. Surprisingly, control plants produced seeds like vernalised plants. Also, there were no difference between control and vernalised plants' seeds in terms of morphology.

Figure 3.18. The appearance of normal spikes and seeds of Kızıltan.

Figure 3.19. The appearance of abnormal spikes of Kızıltan.

The seed settings of explants subjected to different vernalization time at different callus ages can also be showed as the average seed number per spike for control and vernalised plants. The data with standard errors is shown in Appendix H.

When vernalised and control plants were compared in terms of average seed numbers, there were no significant differences between calli produced from 4 weeks vernalised explant at different age (p=0.87 for vernalised plants and p=0.40 control plants in 95 % confidence interval) (Demirbaş, 2004). In the same way, there were only difference between control and vernalised plants at 9 weeks old calli when we took into account calli produced from 4 weeks vernalised explants (p=0.11 for 9 weeks old calli in 95 % confidence interval).

The seeds characteristics were affected by vernalization time. During experiments, we recorded the number of seed per spike and average weight of seeds in different plants, obtained from calli produced from explants subjected to 4 ° C for 3 weeks, 4 weeks and 5 weeks.

When we compared the results belong to control plants, there were differences between vernalization times at 6 weeks and 12 weeks old calli (p=0.03 for 6 weeks old calli and p=0.04 for 12 weeks old calli) in terms of average seed number and we reached to a maximum value of 8.25, using 5 weeks vernalised explants. In the same way, average seed number of 3 weeks vernalised explants were significantly different than the others at 6 weeks and 9 weeks old calli in vernalised explants (p=0.1 and p=0.2, respectively).

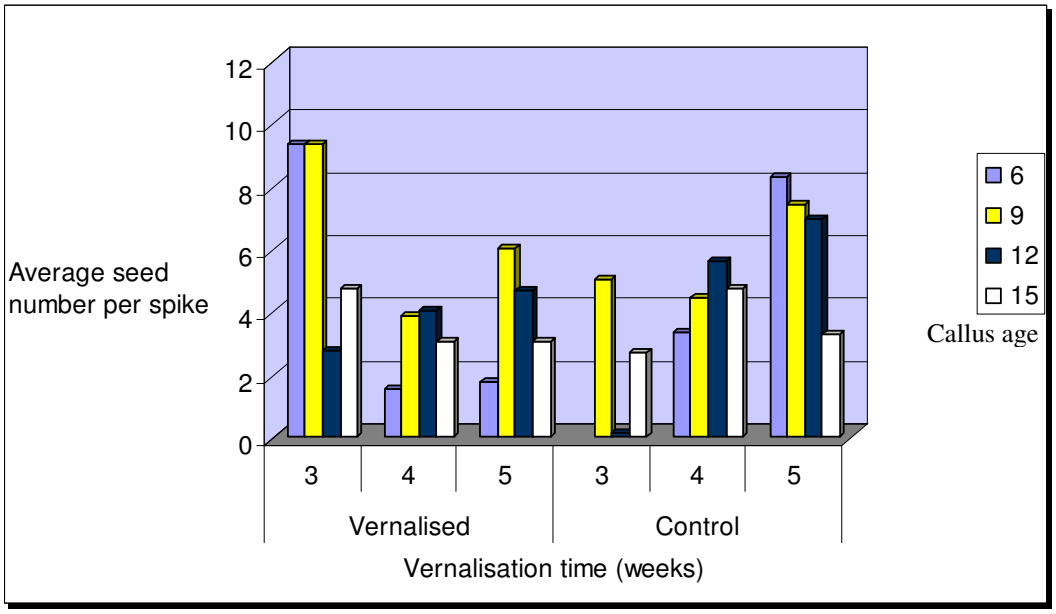

Figure 3.20. Average seed number per spike for Kızıltan (control and vernalised).

In addition to the number of seeds present in spike, their quality is also very important. One of the first parameter associated with seed quality is its weight. As the number and weight of the seeds are one of the traits that determine the grain yield and usually the ultimate goal in wheat production, the regenerated plants are desired to be as good as counterparts which are not originating from tissue culture. The average weights of 1000 seeds of vernalised plants obtained from 6 weeks, 9 weeks, 12 weeks, and 15 weeks old calli range from 32.7 g, 49.9 g, 36.2g, and 48.8 g , respectively. The average weights of 1000 seeds of control regenerated plants change between 28.4 g, 12.9 g, 17.4 g, and 30 g at 6 weeks, 9 weeks, 12 weeks and 15 weeks old calli, respectively. After statistical analysis, average weights of 1000 seeds were significantly different between control regenerated and vernalised plants, obtained from calli produced from 4 weeks vernalised explants, at 9 weeks, 12 weeks, and 15 weeks old calli.

The average weight of 1000 seed of control plants grown in greenhouse was 25.5 (n=202). In literature, 1000 seed weight of Kızıltan was stated as 46-48 g (Central Research Institute for Field Crops, 2002). Our findings from regenerated plants, actually, should not be thought as deviating from literature value which is given as the value obtained in field trials. In green house conditions where the plants are grown in pots, it is expected to obtain grain yield lower than field values.

Another comparison was made between different vernalization times in terms of the average weights of 1000 seeds of regenerated plants for both controls and vernalised plants. Table 3.5 shows average seed weight of control and vernalised plants at different vernalization times. We observed that there were no correlations between vernalization time in terms of average seed weight for both control regenerated and vernalised plants.

Table 3.5. Average seed weight (Kızıltan).

Callus Age	Ver. Time	N (control)	Average weight of 1000 seeds of control regenerated plants (g)	n (ver.)	Average weight of 1000 seeds of vernalised plants (g)
6 WEEKS	3W	NA	NA	317	24.52±0.35
	4W	908	28.36±0.33	405	32.67±0.45
	5W	231	35.67±0.43	339	29.51±0.48
9 WEEKS	3W	275	26.48±0.32	280	32.10±0.54
	4W	610	12.94±0.65	714	49.87±0.56
	5W	141	29.34±0.44	535	28.59±0.78
12 WEEKS	3W	1	32.20±0.00	44	23.54±0.00
	4W	498	17.35±0.33	473	36.24±0.65
	5W	228	41.11±0.54	187	29.79±0.76
15 WEEKS	3W	94	NA	251	46.56±0.33
	4W	398	29.97±O.49	200	48.78±0.56
	5W	134	68.50±0.88	60	29.68±0.87

3.3. *Agrobacterium*-mediated transformation studies

3.3.1. Transformation studies of Kızıltan

3.3.1.1. Effect of transformation protocol along with vernalization time at different callus age

Two transformation protocols were used in transformation of Kızıltan. The first one was MMA based (Mahmoudian et al, 2002) and the second was MG/L based (Amoah et al, 2001; Wu et al, 2003). The preliminary studies on transformation of wheat inflorescence with *Agrobacterium tumefaciens* AGL I were carried out on wheat inflorescence which have been kept at callus induction medium for 7, 14, 21, 28, 35, 42, and 63 days. In these preliminary studies, MMA based protocol were used. In this procedure, bacteria were centrifuged and re-suspended in MMA medium and incubated for 1 hour at 24 ° C prior to inoculation with plant tissues. For other species, this period may not be important because during co-cultivation, active bacteria will have opportunity to transfer its DNA. Since *Agrobacterium*-associated hypersensitivity response is a very big problem in cereals, during the point at which explant is met with bacterial cells, the bacteria should be in its most active and ready state.

Transformations were carried out in MMA inoculation medium including acetosyringone (200 µM) and ascorbic acid (100 mg/L) to cope with hypersensitivity problem. Following transformation process, calli were co-cultivated with bacteria on MMD medium which contains acetosyringone and ascorbic acid for 3-4 days. After the co-cultivation step, calli were incubated on MS_2 medium for callus induction.

In MG/L based transformation protocol, transformations were performed with 7, 14, 21, 28, 35, 42, and 63 days old calli same as MMA based protocol. Co-

cultivation was carried out in CCM medium. Histochemical GUS assays were carried out 9 days after transformation. While recording blue spots, fair blue spots were not taken into account. Figure 3.21 shows fair blue and blue spots obtained from MMA based protocol.

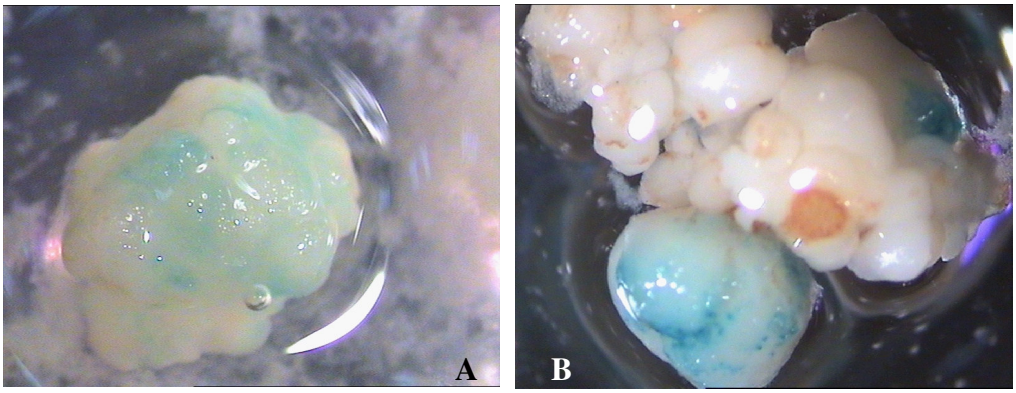

Figure 3.21. Gus activity obtained from MMA based protocol. A. Fair blue B. Blue spots.

When we compare two transformation protocols depending on callus age, we obtained maximum transformation efficiency using 21, 28 and 35 days old calli (Table 3.6). 21-35 days were found to be the period during which plant cells are receptive to *Agrobacterium* infection. Cells can be more prone to transformation not only due to their competence in terms of physiological situation at that developmental stage, but also due to their ability to better cope with the hypersensitivity response when compared to younger explants. In this manner, using immature inflorescence derived calli rather than freshly isolated immature inflorescence seems to be wiser. In the study of Amoah and co-workers, the optimum age of inflorescence derived calli was found as 21 days (Amoah *et al.*,

2001). The difference of our findings with theirs is understandable due to genotypic differences resulting from utilization of different cultivars.

Table 3.6. GUS Frequency with respect to callus age (Data were collected using 4 w vernalization explants).

Callus age (days)	Number of GUS expressed explants* MMA	Number of GUS expressed foci ** MMA	Number of GUS expressed explants * MGL	Number of GUS expressed foci** MGL
7	0/32	0/32	0/44	0/44
14	0/41	0/41	0/34	0/34
21	2/70	2/70	2/20	4/20
28	5/75	10/75	14/121	18/121
35	5/76	8/76	8/60	12/60
42	6/103	6/103	7/91	7/91
63	0/42	0/42	0/65	0/65

(*) Frequency is given as number of GUS expressing explants over total number of explants.

(**) Frequency is given as number of GUS expressing foci over total number of explants

When we compare two transformation protocols, there were significant differences in terms of GUS frequency. Also, we obtained strong transient gene expression by using MG/L based protocol. Figure 3.22 shows GUS expression obtained from two different protocols.

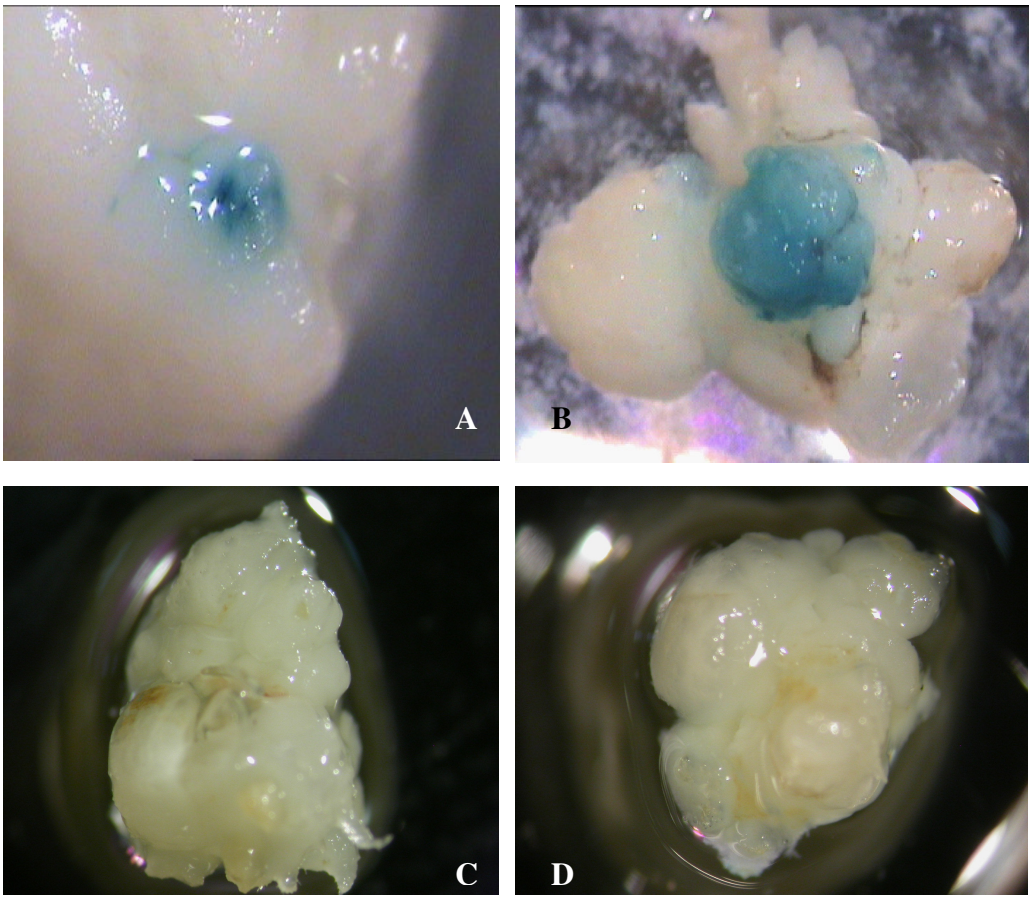

Figure 3.22. GUS expression obtained from different protocols using 4 weeks vernalised explants (Kızıltan). A. MMA based protocol B. MGL based protocol C. Control of MMA based protocol D. Control of MGL based protocol.

We can compare two different protocols results at different callus age depending on vernalization time. When we take into account 3 weeks vernalised explants, GUS frequency obtained from MGL protocol were significantly higher than MMA protocol at 21 days and 28 days old calli and we obtained maximum GUS expression using MGL protocol as 40 % and 20 % at 21 days and 28 days old calli, respectively (Figure 3.23).

Also, GUS expression percentage of two different protocols could be compared using calli, induced from 4 weeks vernalised explant. The results revealed that there were significant differences between MGL and MMA protocols in terms of % GUS foci at 21 days and 35 days old calli.

We also investigated the effect of vernalization time on transformation efficiency. Therefore, we used two different explants, that is, 3 weeks and 4 weeks vernalised, during transformation. Since there were no significant differences between 3 and 4 weeks vernalised plants in terms of regeneration, we determined to use them. Therefore we decreased explants formation time and we had chance to perform more transformations. When the GUS frequencies obtained from MMA protocol were considered, four weeks vernalised explants have higher GUS frequency than three week ones at 28 days and 42 days old calli. However, MGL protocol results revealed that there were only significant differences between two vernalization times at 21 days old calli. Also we obtained maximum GUS frequency, 40 %, by using 3 weeks vernalised explants with MGL protocol at 21 days old calli (Figure 3.23).

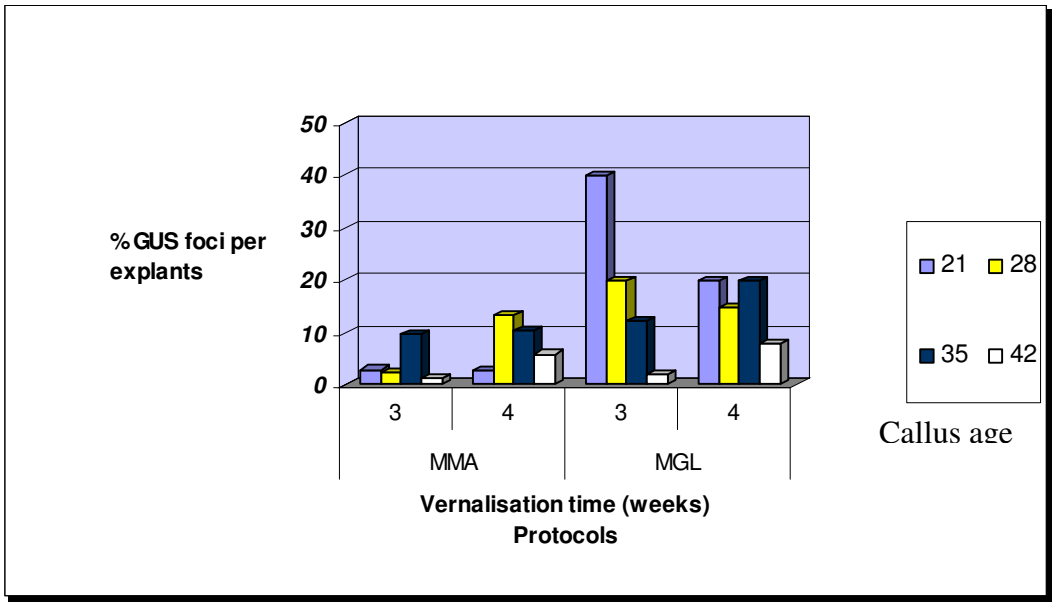

Figure 3.23. GUS expression frequency with respect to two transformation protocols along with different vernalization time (Kızıltan).

3.3.2. Transformation studies on Bezostaja

3.3.2.1. Effects of transformation protocol and vernalization time of explants at different callus age

In preliminary transformations, MMA based protocol were used. However, we did not obtain successful transformation results after 5 trials by using MMA based protocol (Mahmoudian *et al.*, 2002). As a result of unsuccessful transformations, we decided to use another transformation protocols adopted from Wu *et al.* (2003) called MGL based protocol. In this procedure, bacteria were centrifuged and re-suspended in INM medium and incubated 1 hour at 24 ° C prior to explant treatment. All the transformation procedures were same for both protocols apart from medium compositions. Transformations were performed with calli at different ages, that is, 7, 14, 21, 28, 35, 42, and 63 days old.

When we compared two transformation protocols depending on callus age, we obtained maximum transformation efficiency using 28 and 35 days old calli. (Table 3.7).28-35 days were found to be the period during which plant cells were receptive to *Agrobacterium* infection (Figure 3.24).

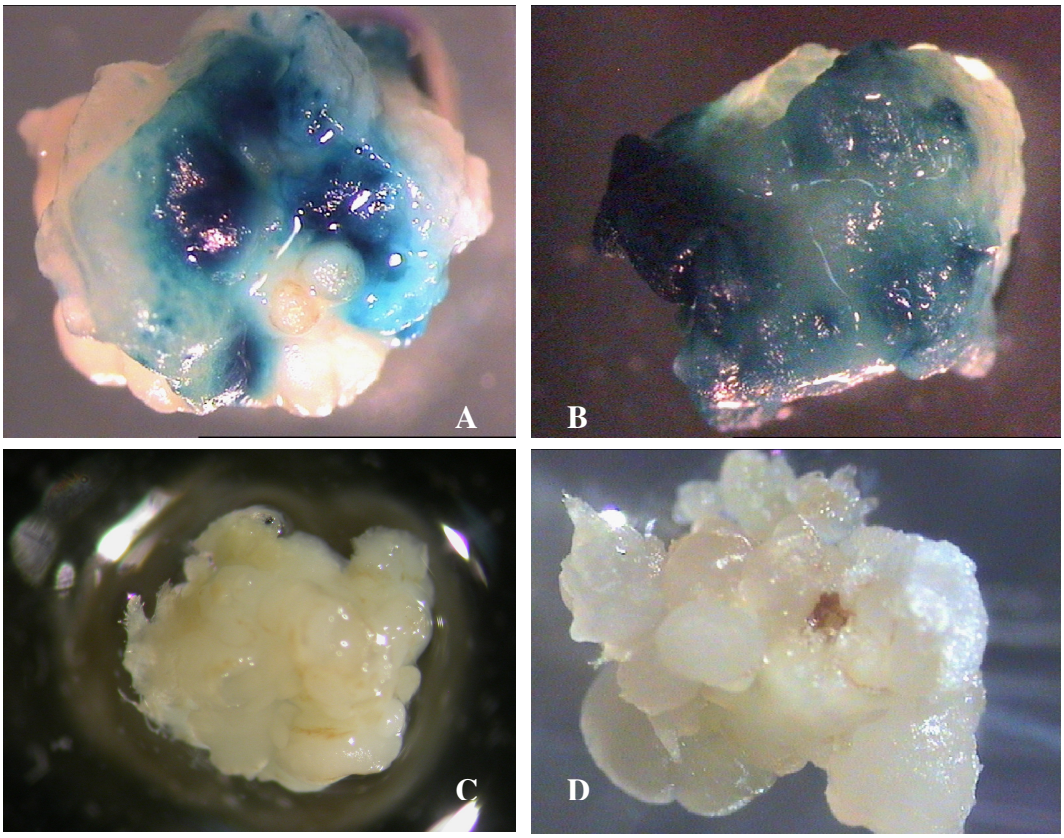

Figure 3.24. GUS expression obtained from different callus age. A.28 days old calli B.35 days old calli C.Control for 28 days old calli D.Control for 35 days calli.

When we look at the results, maximum GUS expression (% GUS foci) were obtained at 28 days and 35 days old calli, 25 % and 75.6 % respectively. The GUS

frequency with respect to callus age of 4 weeks vernalised explants was given in Table 3.7.

Table 3.7. GUS Frequency with respect to callus age (Data were collected using 4 w vernalization explants).

Callus age (days)	Number of GUS expressed explants*	Number of GUS expressed foci**
7	0/56	0/56
14	0/78	0/78
21	3/30	4/30
28	12/48	12/48
35	22/73	55/73
42	8/43	17/43
63	1/64	2/64

(*) Frequency is given as number of GUS expressing explants over total number of explants.

(**) Frequency is given as number of GUS expressing spots over total number of explants

We used two different calli, produced from 4 weeks and 5 weeks vernalised explants, during transformation. We preferred to use 4 weeks and 5 weeks vernalised explants, since these explants produce inflorescence faster than the others.

When we compared the transformation results in terms of vernalization time, the calli, produced from 5 weeks vernalised explants, had more GUS frequency than 4 weeks (Figure 3.25). Further comparison with standard errors is given in Appendix I.

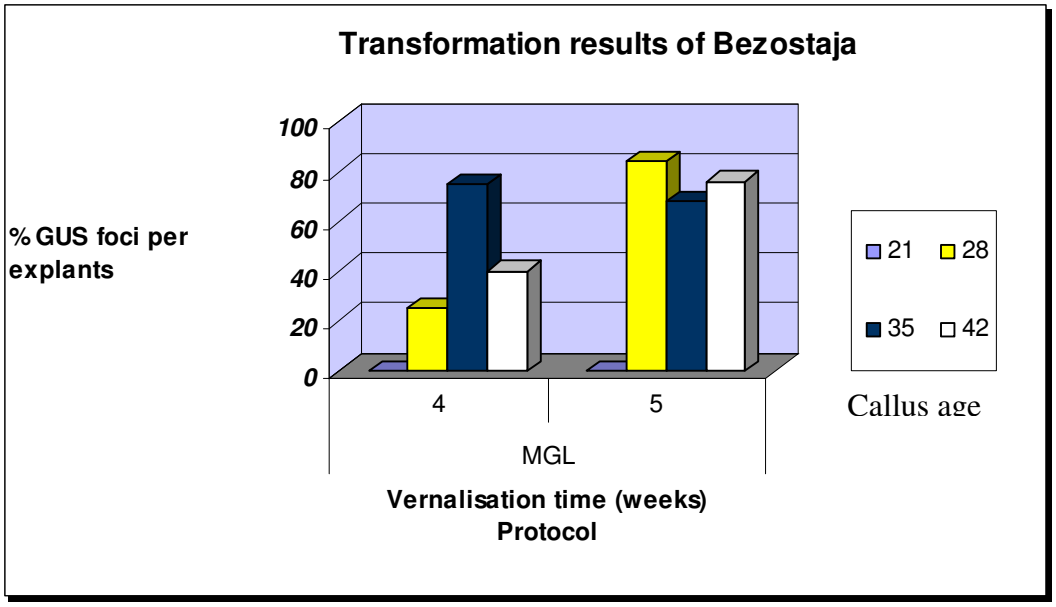

Figure 3.25. GUS expression frequency at different callus age along with vernalization time (Bezostaja).

Although distinct GUS expressions were obtained in most of the trials with MGL protocol, hypersensitivity associated necrosis still persisted as a big problem. The appearances of explants suffering these symptoms are shown in Figure 3.26. In order to obtain increased transformation efficiencies, strategies focusing in relieving these symptoms should be employed. Utilization of antinecrotic agents such as ascorbic acid and cysteine along with silver nitrate which can also prevent ethylene associated symptoms can be suggested.

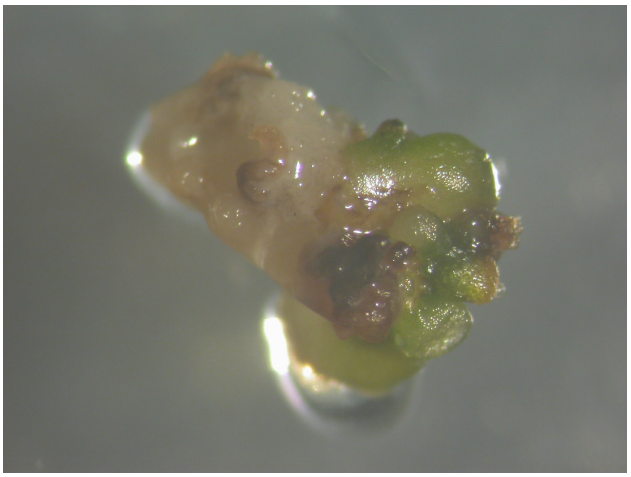

Figure 3.26. Necrosis induced upon transformation procedure.

3.3.3. Determination of selection scheme

Besides having an effective gene delivery system, efficient selection for the transformed cells is extremely important. The plant selection is associated with the incorporated gene *bar* which encodes phosphinothricin acetyl transferase (PAT) and confers resistance to phosphinothricin (PPT) and glufosinate ammonium. Thus, PPT was used as the plant selection agent. In order to determine the optimum concentration of PPT for selection, growth of wheat calli on 0, 3, 6, 10 mg/L PPT was monitored. 35 days old non-transformed calli, which corresponds approximately to the end of co-cultivation duration, were transferred to PPT containing MS_2 medium and callus weight was recorded weekly. Percent changes in callus weight upon different concentrations of PPT applications were demonstrated in Figure 3.27.

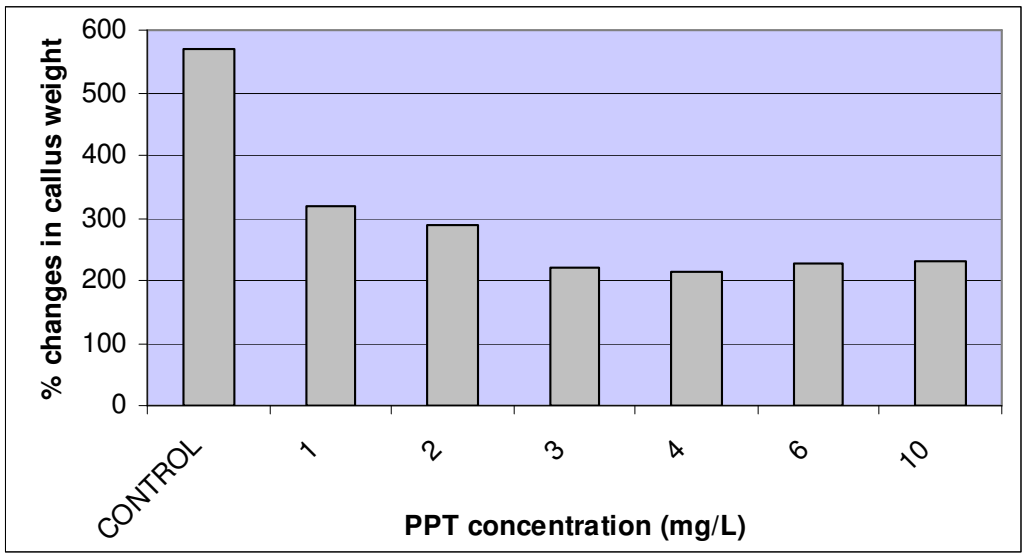

Figure 3.27. Percent changes in callus weight

The graph shows that even with 1 mg/L PPT application callus growth was retarded with respect to control group. At the end of 4 weeks, the cease in growth was more apparent with 10 mg/ L PPT application. According to these results, when constructing selection scheme for Kızıltan and Bezostaja calli, it seems wise to start with 1 mg/L PPT and increase the selection pressure up to 10 mg/L gradually.

In order to remove the *Agrobacterium* cells after transformation, we used Timentin. When we look at the literature, optimum timentin concentration varies between 150 mg/L and 250 mg/L. To evaluate the optimum concentration of timentin in our experimental system, an experiment was designed. Six weeks old calli, the best in terms of regeneration capacity, were transferred into regeneration medium containing 0, 160, 200, and 300 mg/L timentin and we observed the effect of timentin on regeneration. The appearance of calli at the end of 4 weeks was shown in Figure 3.28.

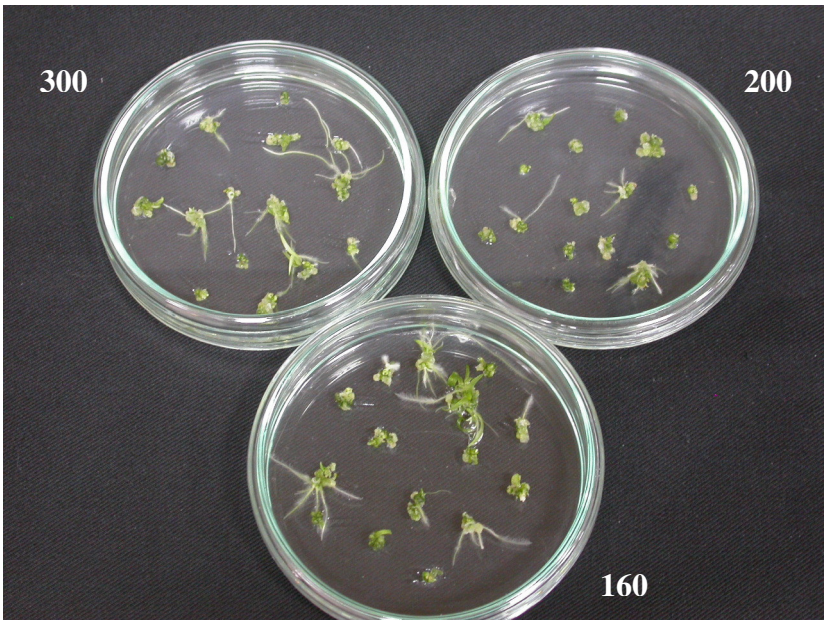

Figure 3.28.The appearance of calli at the end of 4 weeks in regeneration medium at different Timentin concentration.

As shown in Figure 3.28, Timentin did not affect the regeneration at all employed concentrations. As a result, we preferred to use the lowest 160 mg/L Timentin in callus induction and regeneration medium.

We also checked the effect of PPT and Timentin on rooting capacity. In order to monitor this effect, we used regeneration medium containing 160 mg/L Timentin and 0, 1, 2, 3, 4 mg/L PPT. Plantlets were transferred to rooting medium and data was collected at the end of 4 weeks (Figure 3.29). As seen in the Figure 3.29, root formations were ceased at 3 and 4 mg/L PPT. These results seem to be consistent with literature data (Wu *et al.*, 2003 and Przetakiewicz *et al.*, 2004), where similar concentration of PPT was shown to reduce the rooting response.

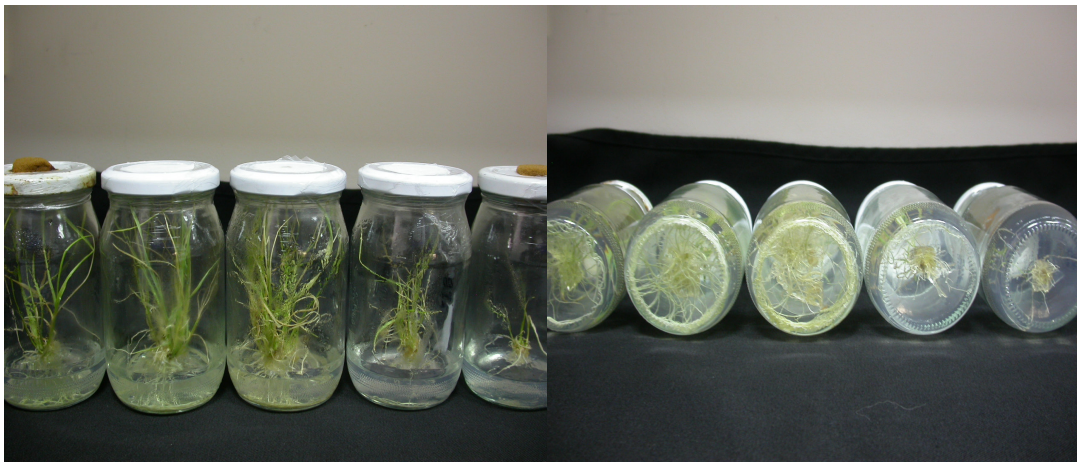

Figure 3.29.The appearance of plantlets containing 0, 1, 2, 3, 4 mg/L PPT and 160 mg/L Timentin at the end of 4 weeks.

Although stable transformants have not obtained yet, current results indicate that even up to 84 % with Bezostaja and 40 % with Kızıltan transient gene expression frequency were observed by the MGL protocol. According to the literature, by using *Agrobacterium* mediated transformation, transient GUS expression frequency was found between 35 % and 65 % in immature embryo derived calli (Khanna and Daggard, 2003), 52.4 % transient GUS activity was reported by Guo and co-workers (1998) with suspension culture derived calli, and a peak value of 76% GUS expression frequency was obtained with 21 day old calli of immature inflorescence (Amoah *et al.*, 2001). It can be said that by using the Turkish wheat cultivars Kızıltan-91 and Bezostaja-01, satisfactory GUS expression frequencies comparable to the literature values were obtained.

CHAPTER 4

CONCLUSION

In this study, the regeneration parameters of immature inflorescence based cultures of winter wheat cultivars Kızıltan (*Triticum durum*) and Bezostaja (*Triticum aestivum*) were optimized. The effects of dark incubation period and vernalization time of explants on regeneration potential were shown.

- Inflorescence formation was affected by vernalization time. We reached maximum percentage of inflorescence formation, 78 %, at 4 weeks vernalised explants in Kızıltan cultivar. However, we observed 5 weeks vernalised explants produce more inflorescence than the others in Bezostaja cultivar, 73%.

- Callus induction rate was reached to 100 % in both of the cultivars irrespective of vernalization time of explants.

- Embryogenic capacity of explants was affected by callus age and vernalization time of explants in Kızıltan cultures. Prolonged dark incubation period decreased regeneration potential. The regeneration potential of 12 weeks and 15 weeks dark incubated explants were found to be significantly lower than that of 6 weeks and 9 weeks. Six weeks and 9 weeks of dark incubation period seems to be optimum in long term transformation studies. Also the regeneration potential of 4 and 5 weeks vernalised explants were found to be significantly higher than 3 weeks

vernalised explants. We reached to maximum regeneration capacity by using 6 weeks old calli produced from 4 weeks vernalised explants (69%).

- However embryogenic capacity of explants was not affected by callus age and vernalization time in Bezostaja cultivar.

- It is evident that longer regeneration time enhances the shooting response. The regeneration potential of Kızıltan inflorescence derived calli was determined as 22 %, 50%, 62 % and 69 % at the end of 4, 8, 12 and 15 weeks of regeneration time, respectively.

- There were no differences between explants belong to different callus age and vernalization time in terms of shoot number during soil transfer in control and vernalised Kızıltan cultivar.

- There were no correlation between callus age and vernalization time of explants in terms of seed number and seed weight in control and vernalised Kızıltan wheats.

- Surprisingly, control plants not subjected to cold after transfer to jar produced morphologically normal seeds and spikes as vernalised plants. The regeneration of plants from immature inflorescences within 260-350 days is possible in Kızıltan cultivar.

Along with these regeneration parameters, due to the less time consuming nature of inflorescences during isolation, reduced growth space and time requirements; Kızıltan and Bezostaja immature inflorescence are suggested as a good candidate in future *Agrobacterium* transformation studies for improvement of Turkish wheat varieties.

In addition to regeneration parameters, *Agrobacterium* mediated transformation protocol for Kızıltan and Bezostaja immature inflorescence tissue were also optimized by monitoring transient expression of *GUS* gene.

- The age of the explant has a supreme importance in transformation success. 21 and 28 days old callus was found to be more successful to *Agrobacterium*- mediated transformation in Kızıltan cultivar. However, 28 and 35 days old callus was found to be more successful to *Agrobacterium*-mediated transformation in Bezostaja cultivar. Using immature inflorescence derived calli rather than freshly isolated immature inflorescence seems to be wiser.

- We observed that vernalization time of explants did not affect the transformation efficiency in Kızıltan and Bezostaja cultivars.

- When constructing selection scheme for Kızıltan and Bezostaja calli, it is suggested to start with 3 mg/L PPT and 160 mg/L timentin and gradually increase PPT upto 10 mg/L.

- *Agrobacterium* associated cell necrosis is still a problem in transformation procedure. In order to obtain increased transformation efficiencies, strategies focusing in relieving these symptoms should be employed.

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APPENDIX A

INFORMATION ON KIZILTAN-91

The agronomic information of cultivar KIZILTAN-91 is summarized in Farmer's booklet of Central Instıute for Crop Plants (2001).

Islah Edildiği Kuruluş	:	Tarla Bitkileri Merkez Araştırma Enstitüsü- ANKARA
Tescil Yılı	:	1991
Sap ve Yaprak Özellikleri	:	Sap 90-95 cm uzunluğunda, yaprakları yeşil renkli tüysüz ve yaprak duruşu yarı yatıktır.
Başak Yapısı	:	Başakları dik duruşlu 7-8 cm. uzunluğunda, yoğunluğu orta sıktır. Kılçıklı olup, hasat olğunluğu döneminde kılçıklarını dökmez.
Dane Özellikleri	:	Dane kehribar renkli, oval yapıda, 8-9 mm. uzunluğunda dır. 1000 dane ağırlığı 46-48 gr. Olup, camsı görünüşlü sert bir yapıya sahiptir. Karın çizgisi dar, derinliği sathi ve yanak şekli yuvarlaktır. Hektolitre ağırlığı 76-78 kg.
Tarımsal Özellikleri	:	Kışa dayanması iyi, kuraklığa dayanması orta iyidir. Erkenciliği orta, verim potansiyeli iyidir. Yatmaya dayanması, gübreye karşı reaksiyonu ve dane dökme mukavemeti iyidir. Harman olma kabiliyeti iyidir.
Hastalık Durumu	:	Sürmeye ve rastığa dayanıklı, sarı pasa töleranslıdır. Kahverengi pasa ise orta dayanıklıdır.
Tavsiye Edildiği Bölgeler	:	Orta Anadolu ve Geçit Bölgelerine tavsiye edilmektedir.

APPENDIX A Continued

The agronomic information of cultivar Bezostaja-01 is summarized in Farmer's booklet of Central Institute for Crop Plants (2001).

Islah Edildiği Kuruluş	:	Rusya'dan getirilmiş ve Anadolu Zirai Araştırma Enstitüsünce adaptasyonu yapılmıştır.
Tescil Yılı	:	1968
Sap ve Yaprak Özellikleri	:	Sap kısa boylu, sağlam yapılı ve gri yeşil renkli olup yaprakları tüysüzdür.
Başak Yapısı	:	Kılçıksız, beyaz kavuzlu, orta uzun, orta sık ve dik başaklıdır.
Dane Özellikleri	:	Sert-kırmızı daneli olup, 1000 dane ağırlığı 40-44 gramdır.
Tarımsal Özellikleri	:	Kışlık bir çeşit olup, soğuğa dayanıklıdır. Ancak kurağa dayanıklılığı azdır. Az kardeşlenir, gübreye reaksiyonu iyidir. Erkenciliği orta olup yatmaya dayanıklıdır. En iyi sonuç sonbaharda erken çıkış sağlandığında alınır. Kardeşlenmesinin düşük olmasından dolayı verim potansiyeli tane ve başak büyüklüğünden kaynaklanır. İlkbahar son donlarından zarar görmez. Ancak yaz kuraklıklarından fazlaca etkilendiği için kır-bayır tarlalar ve yeterli yağış alamayan yörelerdeki alanlar için uygun değildir.
Hastalık Durumu	:	Sarı pasa dayanıklı olup, kara ve kahverengi pasa orta derecede dayanıklıdır. Sürme ve rastığa orta hassastir. Kök ve kök boğazı çürüklüklerinden önemli ölçüde etkilenir.
Tavsiye Edildiği Bölgeler	:	Trakya, Kuzey ve Batı Geçit Bölgeleriyle Orta Anadolunun taban ve sulanabilen alanlarıdır.

APPENDIX B

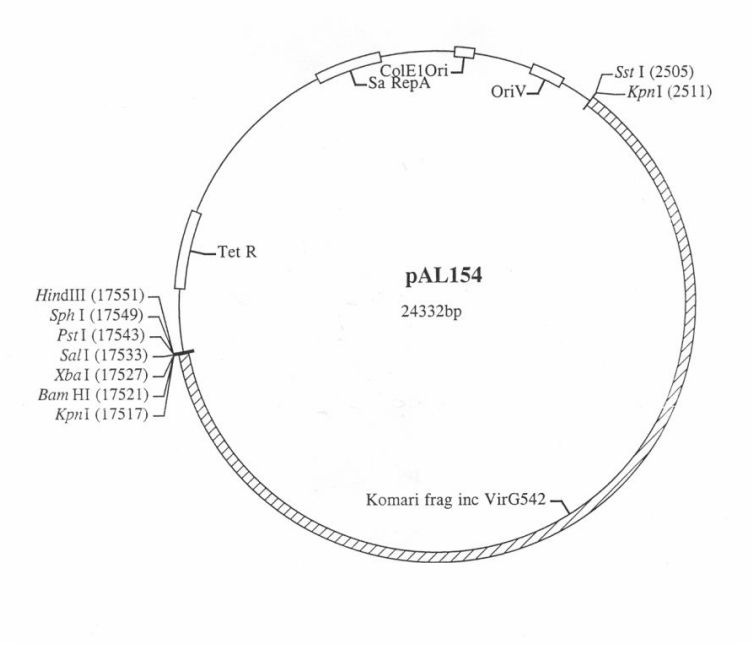
COMPOSITION OF PLANT TISSUE CULTURE MEDIA

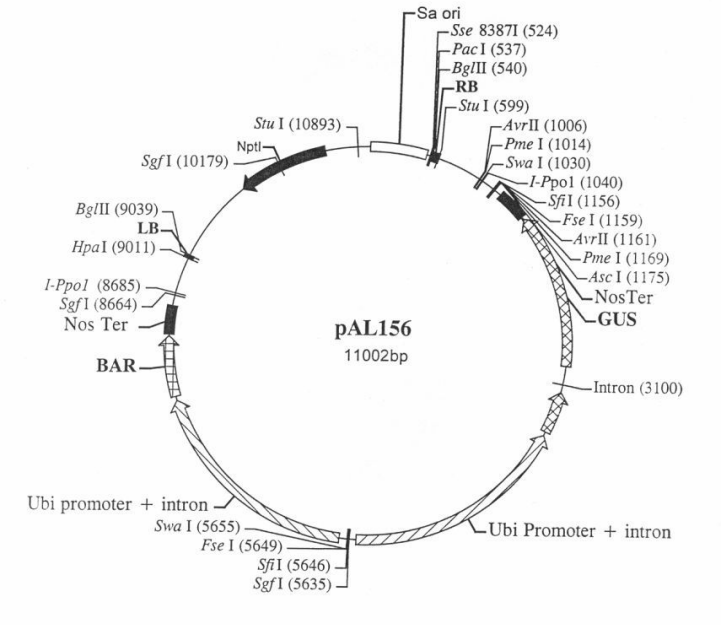
COMPONENT	mg / L
Ammonium nitrate	650.0
Boric acid	6.2
Calciumchloride anhydrous	332.2
Cobalt chloride. $6H_2O$	0.025
Cupric sulfate. 5 H_2O	0.025
Na_2 EDTA	37.26
Ferrous sulfate. 7 H_2O	27.8
Magnesium sulfate	180.7
Manganase sulfate. H_2O	16.9
Molybdic acid (sodium salt). H_2O	0.25
Potassium iodide	0.83
Potassium nitrate	1900.0
Potassiumphosphate monobasic	170.0
Zinc sulfate. 7 H_2O	8.6
Organics	
Glycine (free base)	2.0
Myo-inositol	100.0
Nicotinic acid (free acid)	0.5
Pyridoxine. HCl	0.5
Thiamine. HCl	0.1

4.4 g of powder is utilized to prepare 1 L of medium.

APPENDIX C

PLASMID MAPS





APPENDIX D

TRANSFER AGREEMENT FOR THE PLASMIDS AND BACTERIA

MATERIAL TRANSFER AGREEMENT FOR RESEARCH-ONLY PURPOSES

Huseyin Avni Öktem, an employee of Department of Biological Sciences, Middle East Technical University, 06531 Ankara, Turkey, ('the Recipient') wishes to obtain certain tangible materials and/or information from Wendy Harwood & Matthew Perry of the John Innes Centre as described on the reverse side ('the Materials') for the sole purpose of conducting the research specified on the reverse side ('the Research'). The Recipient acknowledges that all rights to the Materials, whether directly or indirectly enclosed therein as well as extracts, replications, summaries, or derivatives thereof, are the sole property of the John Innes Centre, Norwich Research Park, Colney, Norwich, NR4 7UH, UK ('JIC') and warrants not to use the Materials for any form of commercial exploitation howsoever.

This Material Transfer Agreement does not imply any direct or indirect license or warranty whatsoever with regards to the Material and use thereof nor does it guarantee not to infringe on any rights or claims from third parties with regards to the Material or the Material's suitability, novelty or safety for any purpose whatsoever. In consideration for JIC providing the Recipient access to the Materials and the right to utilise them for the Research, the Recipient agrees to the following conditions:

1. Not to transfer or distribute any part of the Materials or any extracts, replications, summaries, or derivatives thereof to any third party howsoever.
2. Not to use any part of the Materials or any extracts, replications, summaries, or derivatives thereof for any other purpose than the Research.
3. Not to disclose any information whatsoever with regards to the Material and use thereof, without the prior written approval of JIC.
4. To acknowledge the contribution of [Wendy Harwood & Matthew Perry / JIC / the Biotechnology and Biological Sciences Research Council (BBSRC)] in any publication that may result from use of the Materials.
5. To hold harmless JIC and its governors, officers, employees and agents from any and all liabilities or claims brought by third parties resulting from the transfer to and use of the Materials by the Recipient.
6. This Agreement is personal to the Recipient and not capable of assignment.
7. This Agreement is subject to English Law and exclusive interpretation by the English Courts

Please, have (an) authorized officer(s) of the Middle East Technical University signify the Recipients acceptance of the above by signing and dating two copies of this Agreement and return both copies to Mary Anderson, Contracts Manager, John Innes Centre, Norwich Research Park, Colney, Norwich, NR4 7UH, UK. Upon receipt of two completed and executed copies of this Agreement the Materials will be then be sent to the Recipient.

APPENDIX D, CONTINUED

On behalf of and for Middle East Technical University	Huseyin Avni Öktem
Date: March 26, 2003	Date: March 25, 2003
Signature:	
Name (print): AHMET ACAR	HÜSEYIN AVNI ÖKTEM
Title: PROF. DR., VICE-PRESIDENT	PROF. DR

LIST OF THE MATERIALS

Plasmid pAL156, developed in the Crop Genetics department by D. Lonsdale.
pAL156 is an *Agrobacterium tumefaciens* binary vector carrying the BAR selectable marker and the GUS reporter genes.

Plasmid pAL154, developed in the Crop Genetics department by D. Lonsdale.
pAL154 is an *Agrobacterium tumefaciens* binary vector; a derivative of pSoup carrying the Kumari fragment.

DESCRIPTION OF THE RESEARCH

The material will be used in the optimisation of *Agrobacterium*-mediated transformation conditions for local wheat cultivars. Experiments will be conducted on mature & immature embryos, immature inflorescence and callus explants.

APPENDIX E

BACTERIAL CULTURE MEDIA

Yeast Extract Broth Medium (1L)

Nutrient broth	13.5 g
Yeast extract	1 g
Sucrose	5 g
$MgSO_4.7H_2O$ (2 mM)	0.485 g

The pH of the medium was adjusted to 7.2.

YEB + MES Medium (1L)

Nutrient broth	13.0 g
Yeast extract	1 g
Sucrose	5 g
$MgSO_4.7H_2O$	0.493 g
MES (10 mM)	2.132 g

The pH of the medium was adjusted to 5.6. After autoclaving the medium at 121°C for 20 minutes, sterile acetosyringone was added to the medium (final concentration is 20 μM).

APPENDIX E, Continued

BACTERIAL CULTURE MEDIA

MG/L Medium (1L)

Mannitol	5 g
Yeast extract	2.5 g
Glutamic acid	1 g
$MgSO_4.7H_2O$	0.1 g
KH_2PO_4	0.25 g
NaCl	0.25 g
Tryptone	5 g

The pH of the medium was adjusted to 7.0. After autoclaving the medium at 121°C for 20 minutes, sterile Biotin was added to the medium (final concentration is 1 µg/L).

APPENDIX F

HISTOCHEMICAL GUS ASSAY SOLUTIONS

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
Potassium ferrocyanide, pH = 7.0	0.5 mM
X-Gluc	1.0 mM
Triton X-100	0.1 %

GUS Fixative Solution

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

APPENDIX G

AVERAGE SHOOT NUMBER

Table 3.4. Average shoot number during soil transfer for control and vernalised plants**.**

CALLUS AGE	VERNALIZATION TIME	AVERAGE SHOOT NUMBER (CONTROL)	AVERAGE SHOOT NUMBER (VERNALISED)
6 WEEKS	3W	2.00±0.00	4.84±1.13
	4W	5.71±0.84	4.23±0.40
	5W	4.00±0.81	7.01±1.39
9 WEEKS	3W	4.38±1.36	2.49±0.21
	4W	3.45±0.55	4.12±0.95
	5W	3.00±0.00	4.15±0.44
12 WEEKS	3W	2.00±0.00	7.58±1.91
	4W	4.89±0.75	6.34±0.93
	5W	4.77±0.55	4.19±1.72
15 WEEKS	3W	5.16±0.16	6.63±1.60
	4W	6.50±0.63	9.00±0.72
	5W	6.42±1.91	4.75±0.16

APPENDIX H

AVERAGE SEED NUMBER

Table 3.5. Average number of seeds per spike (Kızıltan).

Callus Age	Vernalization Time	Average number of seed per spike (control)	Average number of seed per spike (vernalised)
6 WEEKS	3W	NA	9.32±2.77
	4W	3.33±0.84	1.51±1.19
	5W	8.25±1.23	1.72±1.02
9 WEEKS	3W	5.00±2.30	9.33±1.96
	4W	4.42±1.03	3.84±0.81
	5W	7.42±0.00	6.01±0.96
12 WEEKS	3W	0.13±0.00	2.75±0.00
	4W	5.60±0.80	4.04±0.72
	5W	6.91±0.25	4.68±1.25
15 WEEKS	3W	2.69±0.00	4.74±2.21
	4W	4.74±1.08	3.03±1.11
	5W	3.27±0.70	3.00±0.09

APPENDIX I

STATISTICAL RESULTS

Analysis of Variance for Bezostaja Inflorecence Formation

```
Source     DF        SS        MS        F        P
C16         1    2.5742    2.5742    40.78    0.000
Error      22    1.3889    0.0631
Total      23    3.9630

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  ---+---------+---------+---------+---
2          12    0.0775    0.2306   (----*----)
5          12    0.7325    0.2704                      (----*----)
                                    ---+---------+---------+---------+---
Pooled StDev =   0.2513             0.00      0.30      0.60      0.90

Source     DF        SS        MS        F        P
C18         1    0.6305    0.6305    14.54    0.001
Error      22    0.9539    0.0434
Total      23    1.5844

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  ---+---------+---------+---------+---
3          12    0.2433    0.2545  (-------*-------)
4          12    0.5675    0.1481                    (------*-------)
                                    ---+---------+---------+---------+---
Pooled StDev =   0.2082             0.16      0.32      0.48      0.64



Source     DF        SS        MS        F        P
C20         1    1.4357    1.4357    20.83    0.000
Error      22    1.5165    0.0689
Total      23    2.9522

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  -------+---------+---------+---------
3          12    0.2433    0.2545  (------*-----)
5          12    0.7325    0.2704                    (-----*------)
                                    -------+---------+---------+---------
Pooled StDev =   0.2625                0.25      0.50      0.75


Source     DF        SS        MS        F        P
C22         1    0.1634    0.1634     3.44    0.077
Error      22    1.0454    0.0475
Total      23    1.2088

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  ----+---------+---------+---------+--
4          12    0.5675    0.1481  (----------*----------)
5          12    0.7325    0.2704               (----------*----------)
                                    ----+---------+---------+---------+--
Pooled StDev =   0.2180              0.48      0.60      0.72      0.84
```

APPENDIX I, CONTINUED

Analysis of Variance for Kızıltan Inflorecence Formation

```
Source     DF        SS        MS        F        P
C16         1    0.9923    0.9923    17.15    0.000
Error      22    1.2731    0.0579
Total      23    2.2654

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  ----+---------+---------+---------+--
2          12    0.2667    0.2434  (------*-------)
5          12    0.6733    0.2377                    (-------*------)
                                    ----+---------+---------+---------+--
Pooled StDev =   0.2406              0.20      0.40      0.60      0.80


Source     DF        SS        MS        F        P
C18         1     0.167     0.167     1.56    0.226
Error      22     2.358     0.107
Total      23     2.525

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  -----+---------+---------+---------+-
3          12    0.6108    0.3511   (-----------*-----------)
4          12    0.7775    0.3018             (------------*-----------)
                                    -----+---------+---------+---------+-
Pooled StDev =   0.3274              0.48      0.64      0.80      0.96



Source     DF        SS        MS        F        P
C20         1    0.0234    0.0234     0.26    0.615
Error      22    1.9774    0.0899
Total      23    2.0008

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  -----+---------+---------+---------+-
3          12    0.6108    0.3511   (--------------*--------------)
5          12    0.6733    0.2377         (--------------*--------------)
                                    -----+---------+---------+---------+-
Pooled StDev =   0.2998              0.48      0.60      0.72      0.84


Analysis of Variance for C21
Source     DF        SS        MS        F        P
C22         1    0.0651    0.0651     0.88    0.358
Error      22    1.6235    0.0738
Total      23    1.6886

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  --------+---------+---------+--------
4          12    0.7775    0.3018           (-------------*------------)
5          12    0.6733    0.2377   (------------*-------------)
                                    --------+---------+---------+--------
Pooled StDev =   0.2717                    0.60      0.72      0.84
```

APPENDIX I, CONTINUED

Analysis of Variance for Embryogenic Capacity (Kızıltan)

3 weeks vernalised explants

```
Source     DF        SS        MS        F        P
C1          1    0.0006    0.0006     0.05    0.838
Error       6    0.0815    0.0136
Total       7    0.0821

                                 Individual 95% CIs For Mean
                                 Based on Pooled StDev
Level       N      Mean     StDev  --------+---------+---------+--------
6           4    0.2833    0.0910    (-------------*--------------)
9           4    0.2657    0.1374  (--------------*-------------)
                                 --------+---------+---------+--------
Pooled StDev =   0.1165               0.20      0.30      0.40



Source     DF        SS        MS        F        P
C3          1   0.05167   0.05167     6.78    0.040
Error       6   0.04575   0.00762
Total       7   0.09741

                                 Individual 95% CIs For Mean
                                 Based on Pooled StDev
Level       N      Mean     StDev  ---------+---------+---------+-------
 6          4   0.28330   0.09095               (--------*--------)
12          4   0.12258   0.08353  (--------*--------)
                                 ---------+---------+---------+-------
Pooled StDev =  0.08732              0.12      0.24      0.36


Source     DF        SS        MS        F        P
C5          1   0.03202   0.03202     3.66    0.104
Error       6   0.05253   0.00875
Total       7   0.08454

                                 Individual 95% CIs For Mean
                                 Based on Pooled StDev
Level       N      Mean     StDev  ------+---------+---------+----------+
 6          4   0.28330   0.09095              (----------*-----------)
15          4   0.15678   0.09611  (-----------*----------)
                                 ------+---------+---------+----------+
Pooled StDev =  0.09356              0.10      0.20      0.30      0.40


Source     DF        SS        MS        F        P
C7          1    0.0410    0.0410     3.17    0.125
Error       6    0.0776    0.0129
Total       7    0.1186

                                 Individual 95% CIs For Mean
                                 Based on Pooled StDev
Level       N      Mean     StDev  --+---------+---------+---------+----
 9          4    0.2657    0.1374              (----------*-----------)
12          4    0.1226    0.0835   (----------*-----------)
                                 --+---------+---------+---------+----
Pooled StDev =   0.1137           0.00      0.12      0.24      0.36

Source     DF        SS        MS        F        P
C9          1    0.0237    0.0237     1.69    0.241
Error       6    0.0844    0.0141
Total       7    0.1081

                                 Individual 95% CIs For Mean
                                 Based on Pooled StDev
Level       N      Mean     StDev  ----------+---------+---------+------
 9          4    0.2657    0.1374            (-----------*-----------)
15          4    0.1568    0.0961   (-----------*-----------)
                                 ----------+---------+---------+------
```

```
Pooled StDev =   0.1186                    0.12      0.24      0.36


Analysis of Variance for C12
Source     DF        SS        MS        F        P
C11         1   0.00234   0.00234     0.29    0.610
Error       6   0.04864   0.00811
Total       7   0.05098
                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  ---------+---------+---------+-------
12          4   0.12258   0.08353   (---------------*---------------)
15          4   0.15678   0.09611         (---------------*---------------)
                                  ---------+---------+---------+-------
Pooled StDev =  0.09004                  0.070     0.140     0.210
```

Analysis of Variance for Embryogenic Capacity (Kızıltan)

4 weeks vernalised explants

```
Analysis of Variance for C30
Source     DF        SS        MS        F        P
C29         1    0.0109    0.0109     0.31    0.600
Error       6    0.2143    0.0357
Total       7    0.2253
                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  --+---------+---------+---------+----
6           4    0.5899    0.1400        (--------------*---------------)
9           4    0.5160    0.2277   (--------------*---------------)
                                  --+---------+---------+---------+----
Pooled StDev =   0.1890            0.30      0.45      0.60      0.75

Source     DF        SS        MS        F        P
C31         1    0.1231    0.1231     8.62    0.026
Error       6    0.0857    0.0143
Total       7    0.2088
                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  -------+---------+---------+---------
 6          4    0.5899    0.1400                   (--------*---------)
12          4    0.3418    0.0947  (---------*---------)
                                  -------+---------+---------+---------
Pooled StDev =   0.1195                0.30      0.45      0.60

Source     DF        SS        MS        F        P
C33         1    0.0590    0.0590     2.60    0.158
Error       6    0.1361    0.0227
Total       7    0.1950
                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  -----+---------+---------+---------+-
 6          4    0.5899    0.1400              (-----------*------------)
15          4    0.4182    0.1605   (-----------*-----------)
                                  -----+---------+---------+---------+-
Pooled StDev =   0.1506               0.30      0.45      0.60      0.75

Source     DF        SS        MS        F        P
C35         1    0.0606    0.0606     1.99    0.208
Error       6    0.1824    0.0304
```

```
Total        7    0.2431
                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level        N      Mean     StDev  ----+---------+---------+---------+--
 9           4    0.5160    0.2277            (----------*---------)
12           4    0.3418    0.0947  (----------*----------)
                                    ----+---------+---------+---------+--
Pooled StDev =    0.1744              0.20      0.40      0.60      0.80

Source      DF        SS        MS        F        P
C37          1    0.0191    0.0191     0.49    0.509
Error        6    0.2328    0.0388
Total        7    0.2519
                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level        N      Mean     StDev  ---------+---------+---------+-------
 9           4    0.5160    0.2277         (--------------*--------------)
15           4    0.4182    0.1605  (--------------*--------------)
                                    ---------+---------+---------+-------
Pooled StDev =    0.1970                 0.32      0.48      0.64

Source      DF        SS        MS        F        P
C39          1    0.0117    0.0117     0.67    0.444
Error        6    0.1041    0.0174
Total        7    0.1158
                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level        N      Mean     StDev  -----+---------+---------+---------+-
12           4    0.3418    0.0947  (------------*-------------)
15           4    0.4182    0.1605        (-------------*------------)
                                    -----+---------+---------+---------+-
Pooled StDev =    0.1317              0.24      0.36      0.48      0.60
```

Analysis of Variance for Embryogenic Capacity (Kızıltan)

5 weeks vernalised explants

```
Source      DF        SS        MS        F        P
C15          1    0.0280    0.0280     2.29    0.181
Error        6    0.0735    0.0123
Total        7    0.1016
                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level        N      Mean     StDev  ----+---------+---------+---------+--
6            4    0.6904    0.1031            (-----------*----------)
9            4    0.5720    0.1178  (-----------*----------)
                                    ----+---------+---------+---------+--
Pooled StDev =    0.1107              0.48      0.60      0.72      0.84


Source      DF        SS        MS        F        P
C17          1    0.1965    0.1965    11.95    0.014
Error        6    0.0986    0.0164
Total        7    0.2952
                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level        N      Mean     StDev  ---------+---------+---------+-------
 6           4    0.6904    0.1031                   (-------*------)
```

```
12          4    0.3769    0.1492  (-------*-------)
                                   ---------+---------+---------+-------
Pooled StdDev =   0.1282                 0.40      0.60      0.80


Source     DF        SS        MS        F        P
C19         1    0.1216    0.1216     2.45    0.169
Error       6    0.2982    0.0497
Total       7    0.4198
                                   Individual 95% CIs For Mean
                                   Based on Pooled StDev
Level       N      Mean     StDev  ----+---------+---------+---------+--
 6          4    0.6904    0.1031            (----------*----------)
15          4    0.4438    0.2980   (----------*----------)
                                   ----+---------+---------+---------+--
Pooled StDev =   0.2229              0.25      0.50      0.75      1.00


Source     DF        SS        MS        F        P
C21         1    0.0761    0.0761     4.21    0.086
Error       6    0.1084    0.0181
Total       7    0.1845
                                   Individual 95% CIs For Mean
                                   Based on Pooled StDev
Level       N      Mean     StDev  ------+---------+---------+---------+
 9          4    0.5720    0.1178              (----------*----------)
12          4    0.3769    0.1492  (----------*----------)
                                   ------+---------+---------+---------+
Pooled StDev =   0.1344               0.30      0.45      0.60      0.75


Source     DF        SS        MS        F        P
C23         1    0.0329    0.0329     0.64    0.454
Error       6    0.3080    0.0513
Total       7    0.3408
                                   Individual 95% CIs For Mean
                                   Based on Pooled StDev
Level       N      Mean     StDev  --+---------+---------+---------+----
 9          4    0.5720    0.1178         (-------------*------------)
15          4    0.4438    0.2980  (-------------*-------------)
                                   --+---------+---------+---------+----
Pooled StDev =   0.2266            0.20      0.40      0.60      0.80


Source     DF        SS        MS        F        P
C25         1    0.0090    0.0090     0.16    0.702
Error       6    0.3331    0.0555
Total       7    0.3420
                                   Individual 95% CIs For Mean
                                   Based on Pooled StDev
Level       N      Mean     StDev  ------+---------+---------+---------+
12          4    0.3769    0.1492  (--------------*-------------)
15          4    0.4438    0.2980      (-------------*--------------)
                                   ------+---------+---------+---------+
Pooled StDev =   0.2356               0.20      0.40      0.60      0.80
```

APPENDIX I, CONTINUED

Analysis of Variance for Embryogenic Capacity (Bezostaja)

4 weeks vernalised explants

```
Analysis of Variance for C2
Source     DF        SS        MS        F        P
C1          2    0.0228    0.0114     1.00    0.416
Error       7    0.0802    0.0115
Total       9    0.1031

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  ------+---------+---------+---------+
 6          4    0.2308    0.0455                (---------*----------)
 9          4    0.2280    0.1569               (----------*----------)
12          2    0.1100    0.0141  (--------------*--------------)
                                    ------+---------+---------+---------+
Pooled StDev =   0.1071              0.00      0.12      0.24      0.36
```

5 weeks vernalised explants

```
Analysis of Variance for C4
Source     DF        SS        MS        F        P
C3          2    0.0332    0.0166     1.21    0.362
Error       6    0.0824    0.0137
Total       8    0.1157

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  ------+---------+---------+---------+
 6          4    0.1616    0.0799         (---------*---------)
 9          3    0.2748    0.1773               (----------*----------)
12          2    0.1250    0.0212   (------------*-------------)
                                    ------+---------+---------+---------+
Pooled StDev =   0.1172              0.00      0.15      0.30      0.45
```

APPENDIX I, CONTINUED

Analysis of Variance for Embryogenic Capacity (Bezostaja)
(In terms of vernalization time)

6 weeks old calli

```
Analysis of Variance for C6
Source     DF        SS        MS        F        P
C5          1   0.00957   0.00957     2.27    0.183
Error       6   0.02534   0.00422
Total       7   0.03491

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  ---------+---------+---------+-------
4           4   0.23080   0.04548             (----------*----------)
5           4   0.16162   0.07985   (----------*----------)
                                    ---------+---------+---------+-------
Pooled StDev =  0.06498                 0.140     0.210     0.280
```

9 weeks old calli

```
Analysis of Variance for C8
Source     DF        SS        MS        F        P
C7          1    0.0038    0.0038    0.14    0.726
Error       5    0.1367    0.0273
Total       6    0.1405

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  ---------+---------+---------+-------
4           4    0.2280    0.1569  (-------------*-------------)
5           3    0.2748    0.1773   (---------------*---------------)
                                    ---------+---------+---------+-------
Pooled StDev =   0.1654                 0.15      0.30      0.45
```

12 weeks old calli

```
Analysis of Variance for C10
Source     DF        SS        MS        F        P
C9          1  0.000225  0.000225     0.69    0.493
Error       2  0.000650  0.000325
Total       3  0.000875

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  -----+---------+---------+---------+-
4           2   0.11000   0.01414   (--------------*---------------)
5           2   0.12500   0.02121       (---------------*--------------)
                                    -----+---------+---------+---------+-
Pooled StDev =  0.01803              0.070     0.105     0.140     0.175
```

APPENDIX I, CONTINUED

Analysis of Variance for Shoot Number(Control and Vernalised Plants)

6 weeks old calli

```
Source     DF        SS        MS        F        P
C1          1     17.88     17.88     2.56    0.119
Error      31    216.16      6.97
Total      32    234.03

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  ------+---------+---------+---------+
6          16     5.705     3.385              (-----------*----------)
7          17     4.232     1.663  (----------*----------)
                                    ------+---------+---------+---------+
Pooled StDev =    2.641               3.6       4.8       6.0       7.2
```

9 weeks old calli

```
Source     DF        SS        MS        F        P
C3          1      2.56      2.56     0.38    0.545
Error      21    141.75      6.75
Total      22    144.31

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  -----+---------+---------+---------+-
6          12     3.453     1.933   (------------*------------)
7          11     4.121     3.173        (------------*-------------)
                                    -----+---------+---------+---------+-
Pooled StDev =    2.598              2.4       3.6       4.8       6.0
```

12 weeks old calli

```
Source     DF        SS        MS        F        P
C5          1      7.82      7.82     1.34    0.267
Error      13     75.70      5.82
Total      14     83.51

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  -+---------+---------+---------+-----
6           7     4.893     2.101  (-------------*------------)
7           8     6.340     2.652             (-----------*------------)
                                    -+---------+---------+---------+-----
Pooled StDev =    2.413           3.0       4.5       6.0       7.5
```

15 weeks old calli

```
Source     DF        SS        MS        F        P
C7          1     15.63     15.63     6.85    0.031
Error       8     18.24      2.28
Total       9     33.86

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  ----------+---------+---------+------
6           5     6.500     1.414   (---------*--------)
7           5     9.000     1.600                  (--------*---------)
                                    ----------+---------+---------+------
Pooled StDev =    1.510                      6.4       8.0       9.6
```

6....CONTROL
7....VERNALIZED

APPENDIX I, CONTINUED

Analysis of Variance for Average Seed Number(Vernalised Plants)
(Effect of vernalization time)

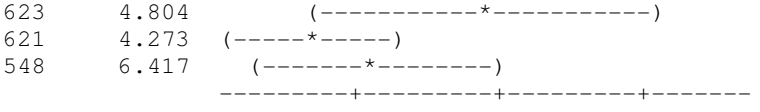
6 WEEKS OLD CALLI

Source	DF	SS	MS	F	P
C1	2	121.2	60.6	2.44	0.114
Error	19	471.2	24.8		
Total	21	592.4			

Level	N	Mean	StDev
3	3	10.623	4.804
4	13	3.621	4.273
5	6	5.548	6.417

Pooled StDev = 4.980

Individual 95% CIs For Mean Based on Pooled StDev



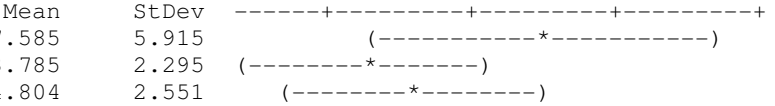
9 WEEKS OLD CALLI

Source	DF	SS	MS	F	P
C3	2	38.8	19.4	1.71	0.211
Error	16	180.9	11.3		
Total	18	219.7			

Level	N	Mean	StDev
3	4	7.585	5.915
4	8	3.785	2.295
5	7	4.804	2.551

Pooled StDev = 3.362

Individual 95% CIs For Mean Based on Pooled StDev



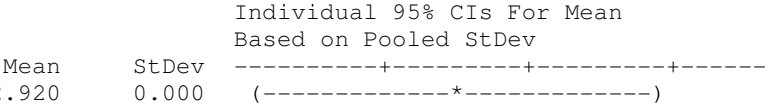
12 WEEKS OLD CALLI

Source	DF	SS	MS	F	P
C5	2	36.1	18.1	1.47	0.302
Error	6	73.6	12.3		
Total	8	109.8			

Level	N	Mean	StDev
3	1	2.920	0.000
4	5	4.158	1.612
5	3	8.127	5.623

Pooled StDev = 3.503

Individual 95% CIs For Mean Based on Pooled StDev



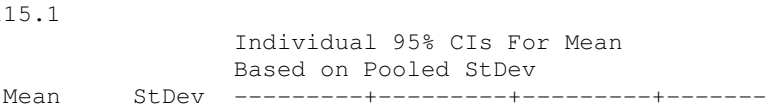
15 WEEKS OLD CALLI

Source	DF	SS	MS	F	P
C7	2	31.5	15.8	1.51	0.278
Error	8	83.6	10.4		
Total	10	115.1			

Level	N	Mean	StDev
3	4	5.808	4.425
4	5	2.554	2.492
5	2	1.791	0.013

Pooled StDev = 3.232

Individual 95% CIs For Mean Based on Pooled StDev



APPENDIX I, CONTINUED

Analysis of Variance for Average Seed Number(Control Plants)
(Effect of vernalization time)

6 WEEKS OLD CALLI

Source	DF	SS	MS	F	P
C13	1	41.33	41.33	5.62	0.035
Error	12	88.22	7.35		
Total	13	129.54			

Level	N	Mean	StDev	Individual 95% CIs For Mean Based on Pooled StDev
4	11	4.043	2.797	(----*-----)
5	3	8.230	2.234	(----------*----------)
Pooled StDev =		2.711		3.0 6.0 9.0 12.0

9 WEEKS OLD CALLI

Source	DF	SS	MS	F	P
C15	2	7.9	3.9	0.22	0.807
Error	15	272.5	18.2		
Total	17	280.4			

Level	N	Mean	StDev	Individual 95% CIs For Mean Based on Pooled StDev
3	6	6.655	5.641	(------*-------)
4	11	6.110	3.366	(----*-----)
5	1	3.630	0.000	(----------*----------)
Pooled StDev =		4.262		-5.0 0.0 5.0 10.0

12 WEEKS OLD CALLI

Source	DF	SS	MS	F	P
C17	2	27.43	13.72	4.92	0.046
Error	7	19.50	2.79		
Total	9	46.93			

Level	N	Mean	StDev	Individual 95% CIs For Mean Based on Pooled StDev
3	1	0.130	0.000	(----------*----------)
4	6	4.728	1.955	(----*---)
5	3	6.177	0.446	(------*-----)
Pooled StDev =		1.669		-3.5 0.0 3.5 7.0

15 WEEKS OLD CALLI

Source	DF	SS	MS	F	P
C19	2	3.61	1.80	0.52	0.632
Error	4	13.96	3.49		
Total	6	17.57			

Level	N	Mean	StDev	Individual 95% CIs For Mean Based on Pooled StDev
3	1	4.290	0.000	(----------*----------)
4	4	4.943	2.153	(-------*--------)
5	2	3.300	0.240	(----------*----------)
Pooled StDev =		1.868		0.0 3.0 6.0 9.0

APPENDIX I, CONTINUED

Analysis of Variance for Average Seed Number(4 WEEKS VERNALISED EXPLANTS)
VERNALISED versus CONTROL PLANTS

6 WEEKS OLD CALLI

```
Source     DF        SS        MS        F        P
C10         1       1.1       1.1     0.08    0.782
Error      22     297.4      13.5
Total      23     298.4

                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  ----------+---------+---------+------
V          13     3.621     4.273  (-------------*-------------)
C          11     4.043     2.797    (--------------*--------------)
                                  ----------+---------+---------+------
Pooled StDev =    3.677                   3.0       4.5       6.0
```

9 WEEKS OLD CALLI

```
Source     DF        SS        MS        F        P
C10         1     25.04     25.04     2.83    0.111
Error      17    150.21      8.84
Total      18    175.24

                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  ---+---------+---------+---------+---
V           8     3.785     2.295   (----------*----------)
C          11     6.110     3.366              (---------*--------)
                                  ---+---------+---------+---------+---
Pooled StDev =    2.973              2.0       4.0       6.0       8.0
```

12 WEEK OLD CALLI

```
Source     DF        SS        MS        F        P
C10         1      0.89      0.89     0.27    0.615
Error       9     29.50      3.28
Total      10     30.38

                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  -+---------+---------+---------+-----
V           5     4.158     1.612  (---------------*--------------)
C           6     4.728     1.955        (-------------*-------------)
                                  -+---------+---------+---------+-----
Pooled StDev =    1.810           2.4       3.6       4.8       6.0
```

15 WEEKS OLD CALLI

```
Source     DF        SS        MS        F        P
C10         1     12.68     12.68     2.29    0.174
Error       7     38.74      5.53
Total       8     51.41

                                 Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  ----------+---------+---------+------
V           5     2.554     2.492  (---------*---------)
C           4     4.943     2.153            (----------*----------)
                                  ----------+---------+---------+------
Pooled StDev =    2.352                    2.5       5.0       7.5
```

APPENDIX I, CONTINUED

Analysis of Variance for Average Seed Number(4 WEEKS VERNALISED EXPLANTS)

Effect of Callus Age

VERNALISED PLANTS

Source	DF	SS	MS	F	P
C10	3	7.3	2.4	0.23	0.878
Error	27	291.3	10.8		
Total	30	298.5			

Level	N	Mean	StDev	Individual 95% CIs For Mean Based on Pooled StDev
6	13	3.621	4.273	(------*-------)
9	8	3.785	2.295	(--------*---------)
12	5	4.158	1.612	(-----------*-----------)
15	5	2.554	2.492	(-----------*-----------)
Pooled StDev =		3.284		0.0 2.5 5.0 7.5

CONTROL PLANTS

Source	DF	SS	MS	F	P
C10	3	24.09	8.03	1.00	0.407
Error	28	224.56	8.02		
Total	31	248.65			

Level	N	Mean	StDev	Individual 95% CIs For Mean Based on Pooled StDev
6	11	4.043	2.797	(----------*----------)
9	11	6.110	3.366	(----------*----------)
12	6	4.728	1.955	(-------------*-------------)
15	4	4.943	2.153	(-----------------*-----------------)
Pooled StDev =		2.832		3.2 4.8 6.4