UNIVERSITY OF ÇUKUROVA

INSTITUTE OF NATURAL AND APPLIED SCIENCES

Ph.D. THESIS

MÜNEVVER DOĞRAMACI-ALTUNTEPE

THE EFFECTS OF GENOTYPE, MEDIUM AND GROWTH CONDITION OF DONOR PLANTS ON THE HAPLOID PRODUCTION FROM ANTHERS OF DURUM WHEAT (*Triticum turgidum* L.) AND CYTOLOGICAL ANALYSES OF REGENERANTS

DEPARTMENT OF FIELD CROPS

ADANA-2000

ÇUKUROVA ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

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Bu tez 2011. 2000 Tarihinde Aşağıdaki Jüri Üyeleri Tarafından Oybirliği ile Kabul Edilmiştir.

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Bu Tez Enstitümüz Tarla Bitkileri Anabilim Dalında Hazırlanmıştır.
Kod No: 593

T.C. ÇUKUROVA ÜNİVERSİTESİ FEN BİLİMLERİ ENSTİTÜSÜ MÜDÜRLÜĞÜ

Prof. Dr. Melih BORAL
Enstitü Müdürü
İmza ve Mühür

Bu proje Çukurova Üniversitesi Araştırma Fonu tarafından desteklenmistir.
Proje No: FDE-97 D 51

The research embodied in this Thesis was conducted at the USDA-ARS, Northern Crop Science Laboratory, Wheat Germplasm Enhancement Project Fargo, North Dakota, USA.

Münevver Doğramacı-Altuntepe was supported by the Technical and Scientific Research Council of Turkey.

ABSTRACT
PH.D.THESIS

THE EFFECTS OF GENOTYPE, MEDIUM AND GROWTH CONDITION OF DONOR PLANTS ON THE HAPLOID PRODUCTION FROM ANTHERS OF DURUM WHEAT (*Triticum turgidum* L.) AND CYTOLOGICAL ANALYSES OF REGENERANTS

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Year: 2000, Page, 135
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Anther culture is being increasingly used in cereal crop improvement both as a source of haploids and for inducing new genetic variation. In the present study, the androgenetic ability of ten cultivars of durum wheat (*Triticum turgidum* L., $2n = 4x = 28$; AABB) has been investigated, using three different growth conditions and four media. From a total of 86,400 anthers cultured, 324 plants were obtained: 248 green and 76 albino. Genotypes, growth conditions, and media significantly affected anther response and callus production; interactions were also significant. Green plant regeneration was influenced significantly by genotype and growth condition, as well as by genotype × growth condition interactions. Albino plant regeneration was affected significantly only by growth condition. Regenerants showed gametoclonal/somaclonal variation. Differences in morphology, growth habit, adult plant height, spike size, and development of spikes at the first nodes were observed. Mitotic and meiotic chromosomes were studied by conventional staining and fluorescent genomic *in situ* hybridization techniques. Chromosome numbers of the regenerants ranged from 14 to 70. All 76 haploid plantlets ($2n = 2x = 14$; AB) were albino. Some of the 28-chromosome regenerants also were albino. Chromosome number in the green plantlets ranged from 28 to 70. Chromosome number also varied in regenerants originating from the same callus. Both intergenomic and intragenomic multivalents were recorded. An interesting feature was the preferential multiplication of B-genome chromosomes, which formed multivalents (trivalents, quadrivalents and hexavalents). We observed several chromosomal abnormalities, which seemed to increase with the level of polyploidy. Translocations, dicentric chromosomes, chromatid breaks and exchanges, and Robertsonian translocations involving the A- and B-genome chromosomes were observed. Chromosome breakages resulting in centric and acentric fragments, as well as telocentrics were also recorded. Chromosome multiplication and structural aberrations induced during culture may constitute the bases of gametoclonal and somaclonal variations.

From the results of the study, it was concluded that to standardize the anther culture technique for durum wheat new media formulations can be examined in future experiments using the successful cultivars for green plant regeneration from this study.

Key words: *Triticum turgidum,* Anther culture, Genotype, Medium, Growth conditions of donor plants.

ÖZ

DOKTORA TEZİ

MAKARNALIK BUĞDAY (*Triticum turgidum* L.) ANTER KÜLTÜRÜNDE GENOTİP, BESİ ORTAMI VE DONOR BİTKİ YETİŞTİRME KOŞULLARININ HAPLOİD BİTKİ ÜRETİMİNE ETKİLERİ VE REJENERANTLARIN SİTOLOJİK ANALİZLERİ

Münevver DOĞRAMACI-ALTUNTEPE

TARLA BİTKİLERİ BÖLÜMÜ
FEN BİLİMLERİ ENSTİTÜSÜ
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Danışmanlar: Prof. Dr. Rüştü HATİPOĞLU
Prof. Dr. Prem P. JAUHAR
Yıl: 2000, Sayfa, 135
Jüri: Prof. Dr. Rüştü HATİPOĞLU
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Anter kültürünün tahıl ıslahında hem haploid hem de yeni genetik varyasyon kaynağı olarak kullanımı artmaktadır. Bu çalışmada 10 makarnalık buğday (*Triticum turgidum* L., $2n = 4x = 28$, AABB) çeşitinin androgenetik kabiliyetleri, 3 farklı büyüme koşulu ve 4 farklı besi ortamı kullanılarak araştırılmıştır. Toplam 86,400 anter kültüre alınmış, 324 bitki elde edilmiştir: 248 yeşil ve 76 albino. Genotipler, büyüme koşulları ve besi ortamı anter reaksiyonunu ve kallus üretimini önemli derecede etkilemiştir; aynı zamanda bunların interaksiyonları da önemli bulunmuştur. Yeşil bitki rejenerasyonu genotipler, büyüme koşulları ve aynı zamanda bunların interaksiyonlarından önemli derecede etkilenmiştir. Albino bitki rejenerasyonu sadece büyüme koşulları tarafından önemli derecede etkilenmiştir. Rejenerantların gametoklonal/somaklonal varyasyon gösterdikleri saptanmıştır. Morfolojik farklılıklar, habitus, bitki boyu, başak büyüklüğü ve ilk boğumlarda başaklar gözlenmiştir. Mitotik ve mayotik kromozomlar geleneksel boyama ve fluorescent *in situ* hybridization teknikleri kullanılarak incelenmiştir. Rejenerantların kromozom sayıları 14 ile 70 arasında değişiklik göstermiştir. Elde edilen 76 haploid ($2n = 2x = 14$; AB) bitkiciğin tamamının albino olduğu saptanmıştır. Bazı albino bitkilerin $2n = 28$ kromozoma sahip oldukları saptanmıştır. Yeşil bitkilerin kromozom sayıları 28 ile 70 arasında değişmiştir. Kromozom sayısı, aynı kallustan rejenere olan yeşil bitkilerde dahi değişmiştir. Hem intergenomik hem de intragenomik multivalentler kayıt edilmiştir. İlginç bir özellik ise multivalentler (trivalentler, quadrivalentler ve hexavalentler) oluşturan B-genom kromozomlarının tercihen katlanması idi. Ploidi seviyesinin artışı ile arttığı görülen değişik kromozom anormallikleri gözlenmiştir. Translokasyonlar, disentrik kromozomlar, kromatid parçalanmaları ve değişimleri, ve A- ve B-genom kromozomları arasında Robertsonian translokasyonları gözlenmiştir. Sentrik ve asentrik parçacıklara neden olan kromozom parçalanmaları, yanında telosentrik kromozomlara da rastlanmıştır. Kültür boyunca oluşan kromozom katlanması ve yapısal değişimlerin gametoklonal ve somaklonal varyasyonları meydana getirebileceği sonucuna varılmıştır.

Bu çalışmadan elde edilen bulgulardan, makarnalık buğdayda anter kültürü tekniğinin standart hale getirilmesi için bu çalışmada yeşil bitki rejenerasyonu başarılı olan çeşitler kullanılarak yeni besi ortamları üzerinde çalışılabileceği sonucuna varılmıştır.

Anahtar kelimeler: *Triticum turgidum*, Anter kültürü, Genotip, Besi ortamı, Donor bitki yetiştirme kosulları.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor Professor Dr. Prem P. Jauhar (USDA–ARS, Northern Crop Science Laboratory, Fargo, North Dakota, USA) for accepting me in his laboratory and for his invaluable guidance throughout the course of this research over the last three years. Without his help and supervision this work would not have been possible. I am also grateful for his patience and critical review of this manuscript several times. The training in classical and molecular cytogenetics that I have received in Dr. Jauhar's lab will be very useful to me when I return to my country.

My special thanks to my advisor in Turkey, Professor Dr. Rüştü Hatipoğlu for his guidance during my course work and for facilitating my placement in the USDA–ARS Wheat Germplasm Enhancement Laboratory in the United States. I truly appreciate him and Professor Dr. İbrahim Genç for nominating me for the scholarship from the Technical and Scientific Research Council of Turkey that made my graduate studies possible. I am grateful to the Council for giving me this opportunity.

I thank the USDA-ARS for additional funding and use of facilities, which made it possible to conduct this research *par excellence*.

I am also grateful to Professor Dr. M. B. Rao for his invaluable help with statistical analyses of my research data, critical discussions, and for his many trips from his office to the USDA. I also thank Dr. Lynn Dahleen for her help with statistical analyses, useful suggestions, and her support as a friend.

Special thanks to Mr. Terrance S. Peterson for his excellent work on figure layouts and illustrations, reviewing the very first drafts of this manuscript, and valuable discussions and suggestions during my research. His dedication to science and knowledge aided my research a lot. I also appreciate and thank him for his support as a friend.

The staff and students of the Wheat Wide Hybrids and Germplasm Enhancement Laboratory made my graduate study a very memorable period of my life. I am thankful for the companionship and many friendships (particularly Polly McMichael, Lisa Bergh, and Christina Finger) that I developed at USDA-ARS Northern Crop Science Laboratory.

Finally, I express my indebtedness to my family for their support and inspiration.

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ABBREVIATIONS

ADP:	adenosine 5′-diphosphate
AMP:	adenosine 5′-monophosphate
ANOVA:	analysis of variance
ATP:	adenosine 5′-triphosphate
bp:	base pair
BSA:	bovine serum albumen
CS:	Chinese Spring
CTAB:	cetyltrimethylammonium
ctDNA:	cytoplasmic deoxyribonucleic acid
cv:	cultivar
DAPI:	4',6-diamidino-2-phenylindole
dATP:	deoxyadenosine triphosphate
DCS:	direct cell sorting
df:	degrees of freedom
DH:	double haploid
DNA:	deoxyribonucleic acid
dNTP:	deoxynucleoside triphosphate
EDTA:	ethylenediaminetetraacetic acid
EtOH:	ethyl alcohol
FAO:	Food and Agriculture Organization of the United Nations
FITC:	fluorescein isothiocyanate
g:	gram
Ga:	gauge
GISH:	genomic *in situ* hybridization
h:	hour
ha:	hectare
IAA:	indole-3-acetic acid

IBA:	indole-3-butyric acid
ICARDA:	International Center for Agricultural Research in the Dryland Areas
L:	liter
LSD:	least significant difference
LSR:	lightly stained region
M:	molar
mg:	milligram
min:	minutes
ml:	milliliter
mM:	millimolar
MS:	mean square
Mt:	metric tonne
mtDNA:	mitochondrial deoxyribonucleic acid
N:	normal
NAA:	naphthalene acetic acid
PAA:	phenylacetic acid
PEG:	polyethylene glycol
pg:	picogram
PI:	propidium iodide
PMC:	pollen mother cell
QTL:	quantitative trait loci
RFLP:	restriction fragment length polymorphism
RNase:	ribonuclease
rRNA:	ribosomal ribonucleic acid
SCE:	sister chromatid exchange
SSC:	sodium chloride/sodium citrate (buffer)
SSDL:	single-seed descent-derived line
TE:	Tris/EDTA (buffer)
TRDA-ARI:	Turkish Republic Department of Agriculture–Agricultural Research Institute

µ:	micro
µM:	micromolar
I:	univalent
II:	bivalent
III:	trivalent
IV:	quadrivalent
V:	pentavalent
VI:	hexavalent
2IP:	6-γ-γ-dimethylallylaminopurine
2,4-D:	2,4-dichlorophenoxyacetic acid
2,4,5-T:	2,4,5-trichlorophenoxyacetic acid

1. INTRODUCTION

The wheat tribe Triticeae contains three important cereals – wheat, barley, and rye. Common bread wheat (*Triticum aestivum* L. $2n = 6x = 42$; AABBDD genomes) is the most important single food source for humankind, with almost 600 million tons of grain produced annually (FAO Web page 2000). Durum wheat or macaroni wheat (*Triticum turgidum* L., $2n = 4x = 28$; AABB genomes) is also an economically important cereal used for human consumption worldwide. It is most commonly used for making macaroni, noodles, and pasta products. Durum wheat is widely grown in several European countries (including Italy, France, Turkey, Romania, and Ukraine), Canada, and the northern great plains of the United States.

Durum wheat is an allotetraploid whose genomes, A and B, were derived from two diploid wild species. The donor of the A genome is *Triticum urartu* Tumanian (Nishikawa, 1983). Although some degree of controversy surrounds the donor of the B genome (see Kimber and Feldman, 1987; Yen et al., 1996), the available evidence shows that *Aegilops speltoides* Tausch is the source of B genome (Sarkar and Stebbins, 1956; Dvořák and Zhang, 1990; Jauhar, 1996; Dvořák, 1998; Wang et al., 1997). The two progenitors hybridized in nature about 10,000 years ago (Harlan, 1992) giving rise to emmer wheat (*T. turgidum* var. *dicoccoides* Körn). Functioning of unreduced gametes ($n = 14$) of both the female (*Ae. speltoides*) and male (*T. urartu*) parents instantly gave rise to tetraploid emmer wheat (see Jauhar et al., 1999). Simultaneous occurrence of a spontaneous mutation led to the origin of the *Ph1* gene that suppresses pairing among homoeologous (genetically and evolutionarily related) chromosomes of the A and B genomes. This phenomenon was vital to the evolution of tetraploid wheats. Durum wheat of today is a domesticated form of emmer wheat.

Since the corresponding chromosomes of the A and B genomes are homoeologous to one another, *Ph1* suppresses intergenomic chromosome pairing so that only homologous partners pair. Thus, genetically enforced diploid-like pairing forms the basis of disomic inheritance and confers meiotic regularity and hence reproductive stability to tetraploid wheat. Addition of the D genome (from *Aegilops tauschii* Coss.) to tetraploid wheat led to the origin of hexaploid bread wheat (*T.*

aestivum) with genomes AA, BB, and DD. Thus, durum wheat is a forerunner of bread wheat. Superior genes incorporated into durum can be transferred to bread wheat relatively easily.

Since the wheats occupy a unique place in the global agricultural economy, they have been subjected to extensive research particularly on the use of breeding techniques to bring about improvement in yield, quality, and other agronomic traits. Traditional plant breeding deals with the exploitation of existing genetic variability and with the generation, manipulation, and combination of new variability to produce superior plant types most useful to man. Although conventional plant breeding has made remarkable contributions to wheat improvement, the more recent tools of generating new variability can help accelerate wheat breeding programs.

Cell and tissue culture techniques such as anther culture have attracted considerable attention as supplementary tools to cereal crop improvement (Vasil and Vasil, 1994). Anther culture has important applications in wheat improvement both as a source of haploids (Baenziger, 1996; Stober and Hess 1997) and for induction of new genetic variability (Hu, 1997). Anther culture involves the induction of embryoid formation from immature pollen and subsequent regeneration of embryoids into plantlets. Since Ouyang et al. (1973) reported the *in vitro* regeneration of plants from pollen, and Craig (1974) obtained haploids ($2n = 3x = 21$) from anthers of common wheat, the research on wheat anther-culture has progressed rapidly. Anther culture-derived haploids have been used to produce homozygous lines, which accelerate breeding programs (Kasha et al., 1990; Khush and Virmani, 1996). The application of modern methods of direct gene transfer into crop plants also depends on the development of efficient systems for regeneration of full plants from cultured cells and tissues (Vasil and Vasil, 1994; Jauhar and Chibbar, 1999; Dahleen et al., 2000). The development of efficient systems for regenerating haploid callus cultures may enable direct gene transfer into durum wheat. Chromosome doubling after the incorporation of a transgene(s) should facilitate its stable integration into the durum genome.

Anther culture is also widely used as a means of generating new variation. Several factors influence the frequency of green regenerants from anther culture.

Although earlier workers obtained low frequencies (0.7%) of green plants (Ouyang et al., 1973), the frequency of microspore embryogenesis in common wheat has been improved considerably (see Chu et al., 1990; Kasha et al., 1990; Orshinsky et al., 1990). Several factors are considered important for increasing the induction frequency of green plantlets from anther culture (see Hu, 1997; Orshinsky and Sadasivaiah, 1997); these include the genotype of the donor plant and environmental conditions under which these plants are grown (Jones and Petolino, 1988; Lu et al., 1991; Ghaemi et al., 1995; Sibikeeva and Sibikeev, 1996). However, not much is known about the anther-culture response (i.e., the ability to induce embryoids from microspores) of durum wheat. In one case, two green plants were obtained (Hadwiger and Heberle-Bors, 1986), while in another study three green and two albino plantlets were regenerated from anther culture (Ghaemi et al., 1993a). For anther culture to be useful in durum breeding, a large number of anther culture-derived regenerants must be obtained.

The objective of this research was to standardize efficient techniques of durum anther culture for extracting haploids and other green regenerants. Effects of genotype of donor plant, growth conditions, and media on anther culture were studied. These data, along with cytological characteristics of anther culture-derived haploids, polyploids, and aneuploids are reported, and possible chromosomal bases of gametoclonal / somaclonal variation are discussed.

2. LITERATURE REVIEW

Anther culture is being increasingly used in plant breeding programs in two important ways. Its traditional and most common use has been as a source of haploids, which in turn help to accelerate breeding programs. Another important use of anther culture is to generate new genetic variation, which may be exploited in crop improvement programs. Extensive research has been done to improve the methods of anther culture and to extend its uses to various areas of biological research.

Haploids are autonomous, sporophytic plants that have the gametophytic chromosome number because they originate from a gametic cell in the embryo sac or from pollen grain. The haploid embryo can arise from an egg cell (gynogenesis) or from a gametophytic cell other than egg cell (apogamy) or a male gamete (androgenesis). It can also originate from the microspore nucleus before first pollen grain mitosis when pollen grains or whole anthers are cultured *in vitro*. Several mechanisms are known to result in haploid plants. These are mainly classified into three categories: parthenogenesis and apogamy, chromosome elimination and somatic reduction, and *in vitro* culture (Khush and Virmani, 1996).

Since Blakeslee and coworkers first observed haploidy in *Datura stramonium* L. (Blakeslee et al., 1922), several haploids were reported in the following few decades. However, the research and utilization of haploids remained limited because of their low frequency. From the cytogenetic and breeding standpoints, two comprehensive reviews are available on haploid plants (Kimber and Riley, 1963; Magoon and Khanna, 1963). Even until the early 1960s, the occurrence of haploids was reported in only 71 species of angiosperms belonging to 39 genera and 14 families (Kimber and Riley, 1963). However, not long after the discovery of anther culture as a tool for inducing haploids (Guha and Maheshwari, 1964), haploids were induced in 247 species of angiosperms belonging to 88 genera of 34 families (Maheshwari et al., 1983). While summarizing the state of efficiency of different methods of producing haploids, Jensen (1986) stated that anther culture was the most widely used.

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were obtained in *Triticum aegilopoides* (Link) Thell., *T. dicoccoides* Körn. ex Asch. & Graebner, but anthers of *T. aestivum* did not respond. Plants from wheat pollen were first obtained by Ouyang et al. (1973). At that time the regeneration frequency of green plants of bread wheat was only 0.7%. The success in induction of pollen derived plants through anther culture was achieved in several laboratories in the early 1970s. In these and following years, intensive efforts were made to study the procedural and theoretical aspects of wheat anther culture. The pollen plant induction frequency was increased to a level adequate for wheat researchers to apply the anther-culture technique in breeding programs (Hu, 1997).

In recent years, the frequency of microspore embryogenesis in cereals, mainly barley (*Hordeum vulgare* L.), wheat and rice (*Oryza sativa* L.), has been increased significantly. However, very little is known about the anther-culture ability of the tetraploid wheats. In earlier attempts, the response of the different tetraploid wheat cultivars to androgenesis was very low and few embryoids were obtained in some cultivated varieties (Foroughi-Wehr and Zeller, 1990). Only some albino plants were generated in one study (Zhu et al., 1979), 2 green plantlets in another (Hadwiger and Heberle-Bors, 1986), and five green plantlets from yet another study (Ghaemi et al., 1993b). Thus, the problem of embryoid formation and their development into green plantlets in tetraploid wheats was very serious (Hadwiger and Heberle-Bors, 1986; Foroughi-Wehr and Zeller, 1990).

2.1.1. Factors Influencing Anther Culture

2.1.1.1. Genotype

Anther culture is strongly influenced by genotype of donor plant. The response of genes to chemical and physical factors under culture conditions is not yet known (Sopory and Munshi, 1996). To evaluate the genetic component of response and for possible improvement of androgenetic performance by selection, Deaton et

al. (1987) examined the anther-culture response of three spring wheat cultivars. They used three parental lines, three possible F_1 hybrids among the three lines, their F_2s and backcrosses, and evaluated them for callus production and regeneration capacity. They found significant variation for callus formation among different generations of the three crosses. Genetic variation for regenerability was nonsignificant. Callus production was negatively correlated (-0.24) with regeneration capacity. They reported that the random variation in the study was too great to determine whether major-gene differences for anther-culture response existed among the lines. When the material was evaluated for quantitative gene effects, the estimates for the additive gene effects were generally greater than the estimates for the dominance gene effects for callus formation.

Lazar et al. (1987) investigated the anther-culture of alien addition lines of cultivar (cv.) Chinese Spring (CS) with rye and found that chromosome 4R of rye promoted the anther response, while chromosome 6R and 1R affected the regeneration ability positively. By analysis of anther-culture response of a CS/Cheyenne substitution line, Szakács et al. (1988) found that the induction of callus, regeneration of pollen plants and regeneration of green plants were controlled by multigenes. They were found to be independently inherited characteristics affected, respectively, by 7A and 1B, and 3A and 2D chromosomes.

In a further study Lazar et al. (1990) reported that both genetic and environmental factors influenced callus formation and plant regeneration in wheat anther culture. Interactions among these factors were also significant, although genotype was a major determinant of callus and embryo production.

Agache et al. (1989) reported that the genetic factors are one of the major contributors to *in vitro* response of cultured wheat tissues. Using CS monosomic 1D, single chromosome CS substitution lines of chromosome 5B or chromosome arm 5BL, and F_1 hybrids heterozygous for the 1B chromosome structure (1BL.1BS/1BL.1RS), they studied anther-culture response. Genes on 1D and 5BL increased embryo frequency; gene(s) involved in regeneration ability were located on the 1RS, and a gene increasing albino frequency was located on 5B of CS. The authors, therefore, concluded that a few genes with major effects were involved in

the determination of anther-culture response.

Sagi and Barnabas (1989) studied the cytoplasmic control of *in vitro* microspore embryogenesis in the anther-culture of hexaploid wheat. They used two sets of seven lines of wheat with different cytoplasms, the euplasmic nucleus donors, as well as six alloplasmic lines derived from wild species of the genera *Triticum* and *Aegilops*. Significant cytoplasmic and nuclear effects, but no cytoplasmic-nuclear interaction, were found for embryogenic anther response. The cytoplasmic background of the lines cultured did not affect plant regeneration significantly.

Foroughi-Wehr and Zeller (1990) investigated the anther-culture ability of 21 hexaploid spring wheat cultivars, four durum wheat cultivars, and 50 hexaploid winter wheat cultivars. The callus and embryo frequency varied with the genotype. In general, a higher frequency of responding anthers was observed in winter cultivars. The authors obtained green plants in 15 spring and 31 winter wheat cultivars. The regeneration rate was particularly high in winter varieties that carried the 1B·1R wheat-rye translocation. None of the spring varieties carried a 1B·1R wheat-rye translocation, but genotypic differences were still found within these cultivars. There was embryogenetic response from durum cultivars, but no plants were obtained. The gene controlling haploid induction in alloplasmic wheat lines was located on the rye segment of the 1B·1R translocation chromosome. However, some cultivars (e.g., Apollo, Kristall), even with the 1B·1R translocation, showed low androgenetic ability. The authors suggested that in addition to the 1B·1R translocation, there are other genetic factors that influence the process of microspore embryogenesis. More recently, it was found that induction frequency of pollen callus and green plantlet regenerants was much higher in spring varieties than in winter varieties (Hu, 1997).

Pauk et al. (1991) examined the F_2 populations of hexaploid spring wheat and their parents to compare P-4 and C-17 media for the formation of embryo/callus and to demonstrate a new plant regeneration system. P-4 induction medium was found to be significantly better than C-17 in the number of responsive anthers (RA) and calli induced (CI) at the 1% and 0.1% level, respectively. Genotypic effect was also significant. The yields of RA and CI of F_2 populations were significantly higher than

those of their parents in both media. The existence of heterosis for RA and CI was also reported in the F_2 populations. However, the ratio of green/albino plant regeneration was more favorable in the C-17 derived embryo/calli than in the P-4 derived ones.

Ghaemi et al. (1993a) studied the genetic variability of anther-culture response of tetraploid and hexaploid wheats. A high genetic variability for anther-culture response in eight tetraploid and two hexaploid wheat genotypes was identified. In another experiment, Ghaemi et al. (1993b) investigated the anther-culture ability of 10 tetraploid wheat genotypes and two backcross lines, using two different treatments for anther culture. Their first medium was the potato 2 medium modified by adding 0.5 mgL^{-1} glutamine and solidified with gelrite (4 gL^{-1}), and cultures were incubated in light at 26°C with a 16 h day length. The second medium differed from the first in sugar (maltose) content and consistency (semi-liquid by ficoll), and cultures were incubated in the dark at 28°C. From a study of about 1300 anthers per genotype and treatment, the authors showed that both genotype and treatment affected embryo formation. The backcross lines exhibited significant difference in androgenic abilities when compared to their common parent; five green plantlets were regenerated and fertile doubled haploid (DH) lines were obtained from three out of 12 genotypes studied.

Ghaemi and Carafe (1994) studied the androgenetic response of tetraploid wheat genotypes (AABB) with the addition of the D genome from *Triticum tauschii* Coss. They reported the genetic variability for all steps of the androgenetic process: embryo induction, plant regeneration and the ratio of green and albino plantlet formation. The D-genome addition from *T. tauschii* to tetraploid wheat *Triticum persicum straminum* Vavilov ex Zhuk. had a positive effect on all the traits studied, but the addition of the same D-genome to tetraploid, *Triticum durum gulab* L. did not have any significant effect on androgenesis of the synthetic hexaploid line. The positive effect of the D-genome on androgenesis in one tetraploid line and the absence of its effect in another were explained on the basis of the effect of each genome (A, B and D) separately, their cytoplasmic effect, and/or their possible interactions.

Later these workers studied the reciprocal substitutions for all chromosomes between the hard red winter wheat cultivars Wichita and Cheyenne to investigate the effects of individual chromosomes, as well as their interactions with the genetic background, on androgenesis (Ghaemi et al., 1995). They included duplicate lines for each chromosome to check background homogeneity. In each experiment they used 14 substitution lines, 14 duplicate lines, and two parental genotypes. They reported that 'Wichita' and 'Cheyenne' differed significantly in embryo yield and green plant regeneration, but not in albino or total plant regeneration. Embryogenesis was influenced by some chromosomes of the A, B, and D genomes; green plant production was influenced by all chromosomes of the A and D genomes except 5D; albino and total plant regeneration were affected by some chromosomes of the B and D genomes. Reciprocal effects were obtained with chromosomes 1A, 7A, 1B, 5B, 1D, and 2D for embryogenesis, chromosomes 2D and 7D for green plant regeneration, and chromosome 2D for total plant regeneration. Moieni and Sarrafi (1995) examined 49 winter wheat genotypes and found that genotype was a major determinant of embryoid production and plant regeneration.

Moieni et al. (1997a) examined 21 F_1 hybrids of hexaploid wheat and their parents for anther-culture ability using two different induction media (CHB and W14). They reported that genetic variation was highly significant for androgenetic response. Genetic components were affected by induction media also. General combining ability was significant for all androgenetic traits, except for albino plant regeneration in both media and total plant regeneration in the CHB medium. Specific combining ability was not significant for the traits studied.

Using genotypes carrying the 1BL·1RS translocated chromosome, normal wheat chromosome 1BL·1BS, and ditelosomic lines DT 1BS and DT 1BL, Henry et al. (1993) studied the effect of the 1RS from rye on plant regeneration of microspore-derived embryos in the anther-culture technique. They found a significant difference in yield of microspore-derived green plants between the genotypes carrying the 1RS and 1BS arms.

Löschenberger et al. (1993) investigated the plant formation from pollen of 13 tetraploid wheat species, and two diploids *Triticum monococcum* L. and *T.*

tauschii. All of the tetraploid wheat species produced embryos, but *T. turgidum*, *T. dicoccoides*, *T. dicoccon* (Schrank) Thell., *T. ispahanicum* Heslot, and *T. durum* were among the most productive species. Developmental stage of the microspores and cold pretreatment highly affected embryo induction. Only 0.7% of the embryos of the tetraploid wheats developed into green plants, while 21.7% developed into albino plants. On average, 97% of the regenerated tetraploid plantlets were albino and only 3% were green. The lack of the D-genome did not reduce embryo induction, although it resulted in poor green plantlet regeneration of all tetraploid species. The workers suggested that poor green plant regeneration was not restricted to the AABB-genome of *T. durum*. However, none of the 10 regenerated plantlets of the DD-species *T. tauschii* were green. The workers compared these results with their earlier results obtained from *T. aestivum* anther culture (Löschenberger and Heberle-Bors, 1992) and suggested that allohexaploid nature conferred an advantage for green plantlet regeneration, possibly due to interaction and compensation between genes on chromosomes of the different genomes. Earlier, some workers (e.g., Szakács et al., 1988; Agache et al., 1989; De Buyser et al., 1992) also pointed out that chromosomes of all three genomes of *T. aestivum* appeared to influence green plantlet regeneration.

Ekiz and Konzak (1994a) investigated the anther-culture response of 44 spring wheat lines and their 14 F_1 crosses. They reported significant genotypic differences especially in callus induction and green plant regeneration. The results from the F_1s suggested additive genetic effects for callus induction predominated, although epistasis and heterosis were also observed in some crosses. Some cultivars had dominant callus-suppressing genes, which were functional in F_1 crosses. Additive, epistasis and heterosis genetic effects were observed for total plant regeneration and green plant yield. The authors proposed that callus induction, plant regeneration, and green plant development were under the control of different genetic mechanisms. Later, Ekiz and Konzak (1994b) studied the effects of nuclear genes and cytoplasm on anther-culture response in wheat by analysis of a diallel cross among four selected genotypes. The majority of estimated genetic variation for anther-culture response components represented the effects of nuclear genes.

Hou et al. (1994) studied the inheritance of anther-culture response of four barley cultivars and their 12 F_1 hybrids under two different growth conditions (field and growth chamber). The traits evaluated were number of embryoids, total number of plants, and green plants regenerated per 100 anthers cultured. They found that genetic effects were significant while environment effects were not.

Hatipoğlu and Doğramacı (1995) studied the anther culture response of ten common wheat cultivars and lines, using two different media and three gelling agents. It was found that anther response and plant regeneration were significantly influenced by genotypes, media and gelling agents. The mean number of anther response and plant regeneration varied among the genotypes (1.4-15.5 and 1.2-15.4 per 100 anthers cultured, respectively).

In a further study Hatipoğlu et al. (1998) investigated the anther culture ability of common wheat using seven F_1 hybrids. They reported that 1.67% of the cultured anthers formed calli and/or embryoids, and as a total of 238 green plants and 10 albino plants were obtained. Both callus and/or embryoid induction and plant regeneration were significantly influenced by genotype, the mean number of green plantlets per 100 anthers ranged from 0.06 to 4.07.

Otani and Shimada (1995) investigated the effect of medium on pollen embryo formation and cultured four cultivars of common wheat and eight genotypes of tetraploid wheat (*T. dicoccum*, *T. durum*, *T. persicum*, and *T. turgidum*) on four media (C17, W14, MN6 and Potato-2). Varietal differences were obtained in the pollen embryo formation on each medium. C17 medium containing 0.26 M maltose proved the most effective for the pollen embryo formation in tetraploid wheat, while W14 medium containing 0.26 M maltose was the most effective for common wheat. Green plants were obtained in all four cultivars of common wheat and three genotypes of tetraploid wheat (*T. dicoccum* var. *farrum*, *T. durum* var. *agricunum* and *T. persicum* var. *stramineum*). The frequencies of green plant regeneration were very low in the three tetraploid wheats (0.9, 9.5 and 2.0%, respectively), while three of the four common wheat cultivars (BW2559, Glennson-81 and Seri-82) had a high yield of green plants (22.2, 23.6 and 28.7%, respectively). In the other genotypes of tetraploid wheat, only albino plants were observed. They reported that albinism was

a limiting factor in the use of anther culture of tetraploid wheat.

Wang et al. (1996) studied six DH lines, derived by anther-culture from octoploid triticale × wheat hybrids, using cytological, biochemical and molecular techniques. They reported that lines varied in wheat and rye genome composition, and were either wheat-rye chromosome multiple-addition lines or spontaneous substitutions and/or wheat-rye translocations. Most of the lines contained a pair of 4R chromosomes, although 1R or 7R were present in some. There was instability in the DH lines, particularly with respect to chromosome number. The authors suggested that it was possible to produce alien (wheat/rye) addition, substitution, and translocation lines directly from the anther-culture of intergeneric hybrids.

Ben Amer et al. (1997) studied loci associated with somatic tissue culture ability on chromosome 2B in hexaploid wheat. Three quantitative trait loci (QTL) for tissue culture response (*Tcr*) were mapped on chromosome 2B using single chromosome recombinant lines. They found that *Tcr-B1* and *Tcr-B2*, affecting both green spot initiation and shoot regeneration, mapped in relation to restriction fragment length polymorphism (RFLP) markers in the centromere region and on the short arm of chromosome 2B, linked to the photoperiod-response gene *Ppd2*. A third QTL (*Tcr-B3*), influencing regeneration only, was closely related to the disease resistance locus *Yr7/Sr9g* on the long arm of chromosome 2B.

Holme et al. (1999) studied the anther and microspore culture response of wheat lines from northwestern and eastern Europe. Environments and genotypes significantly affected embryo formation and plant regeneration. On an average, eastern European wheat lines produced 3.6 green plants per 100 anthers, while northwestern European lines yielded only 0.4 green plants per 100 anthers. The ability to regenerate green plants was widespread in both groups. Compared with the anther-culture response, the isolated wheat microspore culture gave on an average 3.7-fold increase in green plants per anther in 85 of these wheat lines.

Manninen (2000) investigated the association between anther-culture response and molecular markers on chromosomes 2H, 3H and 4H of barley. They studied segregation of 111 markers in an androgenetic barley progeny and constructed linkage map. Thirty-one lines were tested for their anther-culture ability.

Associations between molecular markers and anther culture were established. It was reported that two regions on chromosomes 2H and 4H were associated with anther response, three regions on chromosomes 2H (two areas), and 3H were associated with plant regeneration rate, and one region on chromosome 4H was associated with spontaneous doubling.

2.1.1.2. Donor Plant Growth Conditions

Work conducted by Ouyang et al. (1987) proved that conditions under which donor plants were grown could strongly influence the yield of pollen plantlets. The most suitable culture temperature for anther-culture of field-grown material was about 2°C higher than that of greenhouse-grown material. Thus, in anther-culture of cv. Jinghong 5, the highest yield of pollen plantlets appeared at the culture temperature of 30°C when the anther donor plants were grown in greenhouse compared to 32°C when the donor plants were grown in the field. They found that the anthers of the greenhouse-grown material were not developed as well as those of the field-grown material. It was postulated that the poorly developed anthers might have a weaker metabolism, thus requiring lower culture temperature.

Knudsen et al. (1989) studied the anther-culture response of 17 widely grown varieties and one model variety of barley with one replication from field-grown donor plants and one replication from a growth chamber. Plants were regenerated from 18 varieties and green plants were obtained from 16 of them. On an average, 1.6 green plants were obtained per 100 cultured anthers. Estimated variance components for the formation of embryos/callus from the anthers were dominated by the effects of the genotypes and interactions between plant material and environment. Together these accounted for 60.1 and 17.0% of the total variation respectively, while environment was nonsignificant for this character. Neither genotype nor environment significantly influenced plant regeneration from embryos/callus.

Simmonds (1989) assessed the effect of different growth conditions on anther-culture response of seven winter wheat cultivars. Two different conditions were set up for donor plants: (i) 20°C day/15°C night, and (ii) plants were grown under first conditions and then moved into a 10°C day/5°C night, four weeks before

culture. They reported that lower temperature growth conditions (10°C day/5°C night) for anther donor plants, four weeks prior to the mid-uninucleate pollen stage, improved anther-culture response. They also reported that anthers cultured at 25°C did not yield green plants, but at 30°C microspore callus production was increased 25-fold and green plants were regenerated.

From the work of Foroughi-Wehr and Zeller (1990) stated earlier it was also reported that besides the genotypic effect, the anther response was influenced by the growth conditions of the donor material. Field grown material gave better results than plants grown in the greenhouse.

Orshinsky and Sadasivaiah (1997) examined the effect of three different growth conditions, two plating densities and two genotypes on the anther-culture response of soft white spring wheat hybrids. They found significant differences between growth conditions for anther response and plant regeneration. Anthers from plants grown at high temperature (25°C/18°C) or from plants transferred from low temperature (15°C/12°C) to high temperature (25°C/18°C) generally produced more embryos and green shoots, with a lower frequency of albinos, than did anthers from plants grown at low temperatures. However, plating densities of 10 versus 20 anthers per milliliter had little effect on anther response. They found strong genotypic effects on embryo induction and shoot regeneration among ten hybrids.

2.1.1.3. Pollen Development Stage

The pollen and anther developmental stage at the time of excision and culturing is a very important factor for the induction of pollen plants. From the work of Nitsch (1969), it became evident that pollen responded *in vitro* within a limited period of its development. Anthers containing mid- or late-uninucleate microspores were most suitable for anther-culture in wheat (Ouyang et al., 1973; Pan and Kao, 1978). He and Ouyang (1984) made a precise and systematic study on this subject. They found that in some genotypes, haploid calli and subsequently haploid green plantlets could be obtained from anthers at various stages, from as early as the meiosis stage to as late as the binucleate stage. However, the highest peaks of callus plantlet induction frequency occurred at mid- or late-uninucleate stage in almost all

genotypes, as reported in previous papers.

Hassawi et al. (1990) studied the hard red spring wheat cv. Chris and used a "one-step" anther-culture technique haploid generation medium to study microspore development and callus formation. Cytological observations on microspore development were made 7, 10, 13 and 16 days after the anther was placed on the medium. The first mitotic division of the uninucleate microspore formed two nuclei, which varied in size. Further divisions of the vegetative nucleus produced multicellular pollen grains that continued to grow and then were released from the exine. They suggested that this extensive division of the vegetative nucleus could be the source of embryoid or callus generation.

2.1.1.4. Pre-culture Treatments

Progress in this area stems from the work of Nitsch and Norreel (1973) on cold-treated *Datura* flower buds. Subsequent studies have investigated the application of various stresses, e.g., temperature stress treatment (low/high temperature treatment), ethanol stress, centrifugation, reduced atmospheric pressure, irradiation, photoperiod, and starvation of the flower buds, anthers or pollen prior to culture. In most cases, external stresses are important in determining whether the isolated microspores will undergo embryogenesis or not. Among these, only temperature treatment and starvation have been widely used and appear to give significant results (Sangwan and Sangwan-Norreel, 1996).

Lazar et al. (1990) reported that temperature pretreatment has a significant effect on callus development during anther-culture of wheat. They examined different pretreatments: 4°C for 14 days, 10°C or 25°C for 7 days, and found that 4°C for 14 days had more impact than other treatments for anther response.

Ling et al. (1991) investigated the anther-culture response of wheat to different doses of gamma rays in the highly responsive cv. Grebe and the recalcitrant cv. Kite. In this experiment irradiation was applied to spikes prior to anther-culture. For 'Grebe' both the percent of responding anthers and green plant production were significantly increased after the lowest doses (1-3 Gy). Doses of 7 to 10 Gy inhibited anther-culture response. For 'Kite', however, it was only possible to obtain

androgenic calli and green plants after low dose gamma ray treatments. Similar gamma ray treatments were applied to spikes of responsive barley cultivars prior to anther-culture (Laib et al., 1995). None of the three genotypes investigated showed stimulation of anther-culture response after mutagenic treatments. Even a 1 Gy dose of gamma rays decreased the percentage of responding anthers and plant regeneration. It was concluded that the application of low doses of mutagens prior to anther-culture might have prove useful for haploid production in recalcitrant or poorly responding varieties and species.

Stober and Hess (1997) examined the effect of two different pretreatments (7 days at 4°C and 4 h 40°C) on anther donor spikes of wheat, using non-treated anthers as a control. They reported that cold pretreatment gave significantly better results in embryoid induction and in plant regeneration than heat- or no pre-treatment. Heat pretreatment also had significantly lower regenerants than non-treated controls.

Hansen and Andersen (1998) studied isolated microspores of two DH lines of wheat that were treated during microspore culture with 8 different colchicine concentrations ranging from 3 μM to 3 mM for 24 or 48 h. Untreated control cultures produced on an average 220 embryos per spike (100,000 microspore), 68% of the regenerated plantlets were green, and 15% of the flowering plants were fertile. Using colchicine concentrations around 1 mM, the percentage of fertile plants among the regenerants was increased up to 53%. The highest number of embryos and regeneration rates were observed after 24 h colchicine treatments, while the highest frequencies of green plants and fertile plants were obtained with 48 h colchicine treatments. The highest number of DH plants per spike was found after treatment with colchicine concentrations of 300 to 1000 μM.

Karsai and Bedo (1998) investigated the relationship between anther-culture response and aluminum tolerance in wheat. They used a combination of *in vivo* and *in vitro* selection methods to increase aluminum tolerance in wheat × triticale crosses. They reported that both *in vivo* and *in vitro* aluminum treatments significantly influenced anther-culture response. On induction media containing aluminum, the embryoid induction frequency dropped significantly, but there was an increase in the green plant regeneration. They also found that in seedling tests all

DH lines were more tolerant to aluminum than the parent. The application of *in vivo* aluminum selection, before the start of anther-culture, increased the probability of obtaining DH lines with significantly higher tolerance, compared to the original population.

Sopory and Munshi (1996) reported that physical factors such as culture temperature, light, pH of the culture media, atmospheric conditions of the media, anther density and orientation significantly influenced anther-culture response.

2.1.1.5. Media Components

The culture media represent a major factor for inducing the development of green plantlets from microspores. The available literature does not suggest any one culture medium, which could be applicable to all systems. The requirements vary from genotype to genotype. However, generally there is an agreement that the source and amount of total nitrogen as well as the kind of growth regulators are important factors. The basic media generally used have macro and micro elements, vitamins, sugars and growth regulators (Sopory and Munshi, 1996).

Kao (1981) observed that adding ficoll 400 to increase the density of a culture medium considerably increased the frequency of plant formation. Kao and his colleagues further refined the culture conditions for induction of green plants by anther culture (Kao et al., 1991). They indicated that cells in callus tissues under anaerobic conditions (such as those that exist in submerged cultures in liquid medium) contained lactate and alcohol dehydrogenase. The authors hypothesized that accumulation of lactic acid and other organic acids in the tissue might damage the organelles in the cell and result in the formation of albino plants. Thus, for direct embryogenesis from microspores, an ample supply of a fresh, well-buffered medium, and proper aeration for microspore-derived embryos are essential for obtaining high frequencies of green plants. High ficoll and sucrose levels in liquid medium keep the anthers afloat and trap the microspores in the anthers for a sufficient length of time under a condition of sugar starvation and help induce embryogenesis. Wei et al. (1986) also reported that starvation is essential for stabilization of microspores and eventually leads to androgenesis.

Jones and Petolino (1988) investigated the effects of support medium on embryo and plant production from cultured anthers of soft-red winter wheat. They reported that approximately twice as many embryos were produced when anthers were cultured in a liquid as compared to an agar-solidified medium. The addition of activated charcoal to an agar-solidified medium resulted in a considerable increase in embryo production. However, plant regeneration from embryos produced on charcoal-containing medium was significantly lower than those produced on agar. More than twice as many embryos produced on ficoll-containing liquid medium regenerated plants compared to embryos produced in liquid medium only.

Simonson and Baenziger (1992) investigated the effect of gelling agents on wheat anther and immature embryo culture. They used two differentiation media (P1 and 85D12) gelled with one of the three gelling agents (ficoll type 400, wheat starch, or corn starch). Anthers had significantly greater embryoid-initiation frequencies (number of embryoids per anther) on P1 media with corn (0.67) and wheat starch (1.50) than with ficoll (0.47). Anthers on 85D12 media with ficoll, corn starch, or wheat starch had similar embryoid initiation frequencies (0.51, 0.50, and 0.52 respectively). Embryoids on MS media with Bacto-agar, corn starch, and wheat starch had similar embryoid-regeneration frequencies (0.15, 0.20, and 0.22, respectively). In addition, when wheat starch replaced Bacto-agar in immature embryo cultures, the frequency of responding embryos was unchanged, but the frequency of green plants regenerated increased an average of four-fold.

Hou et al. (1993) studied an alternative gelling agent (gelrite, sea plaque agarose, dextran, wheat starch and cellulose) to ficoll 400. Donor spike cold pretreatment and a 3-day mannitol solution pretreatment were used prior to anther culture in barley. They found that media with ficoll, gelrite and sea plaque agarose were equally effective producing embryoids. Production of embryoids from the dextran-containing medium was intermediate, response from the wheat starch medium was low, and cellulose failed to achieve any anther response. Gelrite was considered a satisfactory and more economical replacement for ficoll when large-scale culture is required, such as for breeding purposes. Cold pretreatment of the donor spikes extended and thereby complicated the anther-culture procedure, but

seemed necessary for successful culture. The quality of the embryoids from the 3-day mannitol pretreatment was poorer as indicated by the lower number of total plants and green plants regenerated. However, the 3-day mannitol treatment did not seem to be a suitable substitute for the 28-day cold pretreatment for anther culture.

Hatipoğlu and Doğramacı (1995) also examined the effect of different media (85D12 and P2) and gelling agents (agar, wheat starch, and corn starch) on anther culture of common wheat. It was found that anther response and plant regeneration were better on P2 than 85D12. The effect of gelling agents was also significant. Higher anther response and plant regeneration were obtained when the media gelled with agar or wheat starch, and both of them were better than corn starch.

Fadel and Wenzel (1990) examined media effects on the rate of green plantlet formation, using four different media (MN6, BAC1, C, and potato-2) for anther-culture of five different winter and spring wheat cultivars. An increased number of embryos and calli were produced on ficoll-containing liquid potato-2 medium, whereas the addition of 0.2 ML^{-1} maltose increased the rate of regeneration. The genotype also had a strong effect on plantlet formation. The improved procedure allowed the regeneration of recalcitrant genotypes.

Zhou, et al. (1991) investigated the effect of culture media components on the percentage of green plants from anther culture of wheat. Anthers of a spring wheat cv. Pavon 76 were cultured on potato 4 (P4) induction media with various modifications. Addition of 200 gL^{-1} ficoll to the liquid P4 medium significantly increased the percentage of green plants even though the final yield of green plants per 100 anthers was lower than that on the liquid medium. A higher concentration of maltose (135 gL^{-1}) produced a significantly higher percentage of green plants than the medium containing 90 gL^{-1} maltose or sucrose. The authors suggested that culture medium affects albinism, indicating that the percentage of green plants from wheat anther-culture can be increased by optimizing medium osmotic potential.

Sorvari and Schieder (1987) reported that polysaccharides such as barley starch also improved the response of barley anthers in culture. Starch was presumably hydrolyzed by amylases produced by the anther wall or by the microspores themselves and serves as a slow release form of glucose. Salts such as

Na^+ and K^+, amino acids, organic acids, etc., could be more easily absorbed by pollen grains due to slow metabolism of carbohydrates. These salts and amino acids in pollen cells are important factors for embryogenesis and regeneration of plantlets.

Hunter (1988) reported that the carbon source in the induction medium had a profound effect on anther-culture response. Maltose and other carbohydrates were found to be superior for green plantlet regeneration from barley anthers. To compare the effects of different carbohydrates on barley anther-culture response, Hunter carried out experiments using various carbon sources, e.g., cellobiose, fructose, glucose, maltose, sucrose, and trehalose. It was found that a high concentration of sucrose and its derivative products (such as glucose and fructose) inhibited the induction of pollen and the formation of young embryos. It was also reported that maltose and sucrose produced different breakdown products, which might be a key to the advantages of maltose. It is possible that maltose is degraded more slowly than sucrose, providing a more readily metabolizable carbon source over a longer period of culture.

In a related study, Last and Brettel (1990) reported that when maltose was used in place of sucrose, embryoid yield was increased three to four-fold in wheat anther culture. Orshinsky et al. (1990) also used maltose instead of sucrose for improving wheat anther culture. They clearly showed that the carbon source could have a significant effect on androgenic response. Maltose was superior to sucrose both for callus induction and green plantlet regeneration for a range of genotypes. The use of maltose also allowed green plantlets to be recovered from genotypes that failed to produce plantlets when their anthers were cultured in medium with sucrose. Meanwhile, the genotypes used in their study displayed an exceptionally high level of green plantlet regeneration.

Chu et al. (1990) examined the effects of monosaccharides (especially glucose) instead of sucrose as the carbon source on wheat anther culture. They reported that pollen embryoid induction frequency in a liquid medium with 0.21 M glucose was 40-750 embryoids per 100 cultured anthers, i.e., 2-10 times higher than that in the medium with 0.21 M sucrose depending on the genotypes used. Fructose or a mixture of glucose, fructose and mannitol also showed improved effects over

sucrose. When embryoids were transferred to a filter-sterilized double layer plant regeneration medium with 0.21 M glucose, but without 2,4-D, 40-90% of the pollen embryoids developed into plantlets, whereas in sucrose-containing medium only 0-30% of the embryoids developed. Compared to glucose, sucrose seemed to suppress wheat pollen embryo development. Sucrose probably induces anther cultures to produce ethylene, which defers *in vitro* pollen embryogenesis.

Kao (1993) found that barley microspores were viable when cultured in a sugarless medium. Adding 2 g of glucose to 1 liter of the media resulted in a significant reduction in the frequency of viable microspores. The frequency of viable microspores was further reduced when 50 g of cellobiose, glucose, maltose, melezitose, raffinose or sucrose were added to 1 liter of the medium containing 2 gL^{-1} glucose. Adding 50 g of melibiose, ficoll, polyethylene glycol (PEG) or a combination of 50 g each of ficoll and PEG to 1 liter of the medium had very little effect on the viability of the microspores. Up to 66% of the viable microspores were able to develop into micro calli in the basal medium supplemented with melibiose, maltose, melezitose, raffinose, ficoll, PEG or a combination of ficoll and PEG. Sucrose, cellobiose and glucose added in large quantities inhibited cell division in microspores and only a very few of them developed into microcalli.

Navarro-Alvarez et al. (1994) also investigated the effect of different sugars on wheat anther-culture. They cultured anthers from three different genotypes on a modified Liang's 85D12 initiation medium with seven sugar combinations (galactose, mannose, maltose, fructose, sucrose, glucose, maltose + glucose) at 0.26 M, 2,4-D (2 mgL^{-1}), NAA (1 mgL^{-1}) and glutamine (254 mgL^{-1}). Wheat starch was used as the medium gelling agent. Averaged over the three genotypes, maltose was the best sugar (105 embryoids/100 anthers), followed by glucose (47 embryoids/100 anthers) and maltose + glucose (37 embryoids/100 anthers). These three sugars were superior to the standard medium sugar, sucrose (24 embryoids/100 anthers), and to fructose (12 embryoids/100 anthers). In general, regeneration media with sucrose was very effective for plant regeneration. The highest albino plants occurred on the regeneration medium with glucose. Regeneration media with maltose and maltose + glucose induced the highest frequency of spontaneous doubling.

To study the metabolism of maltose and sucrose by barley microspores, Scott et al. (1995) cultured freshly isolated microspores for 6-24 h on either [U-^{14}C] maltose or [U-^{14}C] sucrose at 40 mM, and determined the distribution of ^{14}C. The amounts of glycolytic intermediates, ATP, ADP and AMP, in microspores were also measured. They reported that cultures on sucrose differed from those on maltose in that the initial rate of metabolism was faster but declined rapidly. It was argued that microspores cultured on 40 mM sucrose died because they metabolized the sugar more rapidly, became hypoxic and consequently accumulated large quantities of ethanol within the cells. Metabolism of maltose was slower, allowing sufficient oxygen available to cells to survive in culture and undergo embryogenesis.

Several authors have shown the beneficial effects of complex organic substances including casein hydrolysate, coconut milk and exogenously supplied amino acids on *in vitro* androgenesis and plant regeneration (Sangwan, 1983; Schmid et al., 1994).

Marburger et al. (1987) studied the effect of a modified potato medium on anther culture of wheat cv. Chris. They prepared the media with (i) extract of whole potato tubers, (ii) extract of the peel only, and (iii) extract of peeled tubers. A 60% increase in anther induction resulted from the peeled tuber extract medium compared to the whole potato medium. They reported that the removal of the peel from tubers probably reduces the concentration of compounds that may be inhibitory to wheat embryogenesis during anther culture.

Feng and Ouyang (1989) investigated the effects of the nitrogen sources on wheat anther culture. They found that in the differentiation medium NO_3^- affected callus induction and regeneration independent of NH_4^+, but the effects of NO_3^- and NH_4^+ were additive. They also observed that KNO_3 concentration influenced green plant regeneration remarkably; the higher the KNO_3 level used, the higher was the frequency of green plant regeneration. It was shown that 15-20 mML^{-1} was most suitable for obtaining high green plant yield. A range of 2-7 mML^{-1} of NH_4^+ concentration was suitable for callus induction, green plant regeneration, and high green plant regeneration. A medium without NO_3^- or NH_4^+ gave considerably lower results.

Liu and Hu (1989) reported that 2.0 mgL^{-1} of 2,4-D was more beneficial when employed in combination with 0.5 mgL^{-1} cytokinin in anther culture of wheat. Higher concentrations of 2,4-D increased the frequency of calli induction as well as the quality of pollen calli. Subsequently, the green plantlet yields were raised significantly in certain genotypes, especially those with poor anther-culture response or winter types. High 2,4-D levels showed no harmful effect but there was still a strong influence of the genotype.

Ziauddin et al. (1992) studied the effect of the auxin phenylacetic acid (PAA) on wheat anther and barley anther/microspore culture. They reported that with PAA the induction response was not significantly different from the controls but a significantly higher number of green plants were produced in both wheat anther and barley microspore culture. However, in barley anther-culture, using PAA did not bring about any improvements.

Trottier et al. (1993) studied the effect of different media composition on the ability of five spring wheat genotypes to produce embryos and green plantlets in anther culture. They found that the addition of a combination of 19 amino acids significantly increased the yield of embryos and green plants. The mean number of green plants per 100 anthers, for the two genotypes studied, went from 28.2 without amino acids in the medium, to 46.7 with addition of amino acids. They also reported that liquid medium with ficoll was more efficient for anther-culture (9.9 green plants/100 anthers) than solid medium (0 green plants), gelatinous media (2.5 green plants/100 anthers), or liquid media with membrane rafts (0 green plants). In addition, the effect of replacement of sucrose with maltose varied with the genotype of the donor plant. Maltose partially inhibited the androgenesis of three responsive genotypes, while it significantly increased the androgenesis of the recalcitrant genotype. An amino acid × maltose interaction was also found. Amino acids without maltose increased androgenesis, but the addition of maltose to the amino acid-enriched medium eliminated this positive effect of the amino acids.

Orshinsky and Sadasivaiah (1994) investigated the effects of three different media on embryoid induction and plant regeneration from cultured anthers of 10 soft white spring wheat cultivars. Embryoid induction was influenced by donor plant

genotype and induction medium. Green plant regeneration frequency also varied with both genotype and induction medium. Of the ten cultivars tested, nine produced both green and albino plants, whereas the other produced only albinos. The induction medium also influenced the fertility (a measure of spontaneous chromosome doubling) of the anther-derived plants.

Schmid et al. (1994) studied the impact of amino acids on *in vitro* androgenesis in maize (*Zea mays* L.) and wheat. In maize, the androgenic induction was not affected by adding casein hydrolysate or potato extract and the best results were obtained with L-asparagine (30 mgL^{-1}), L-glutamine (250 mgL^{-1}), and glycine (2.5 mgL^{-1}) with maximal values of >400 embryos/100 anthers. Combining the cold treatment with L-proline (125 mgL^{-1}) increased the embryo frequency 2 to 3-fold compared to cold treatment alone. In wheat, 200 mgL^{-1} L-proline or 250 mgL^{-1} L-glutamine in combination with a post-plating cold stress (10°C for 10 days) resulted in a maximum of 162 embryos/100 anthers. However, glutamine, aspartic acid, and asparagines, which proved to be most efficient in maize, resulted in reducing embryo production in wheat.

Cho and Kasha (1995) investigated the roles of calcium on microspore-derived embryo formation and ethylene production in barley (cv. Bruce) and wheat (cv. Chris). The inclusion of calcium as $CaCl_2$ in the medium increased embryo production, at an optimal concentration of 1 mM in barley. The addition of the ionophore A23187 (0.5 μM) with $CaCl_2$ (1 mM) further improved the embryoid formation in both wheat and barley. Because exogenous calcium and ionophore increased ethylene production measured at culture initiation time, it was suggested that the positive action of free calcium on embryoid formation might be achieved through initial ethylene production and stimulation of tissue senescence.

2.1.2. Anther Response and Plant Regeneration

The production of haploid plants from anther-culture involves four independent factors: (1) the induction of calli or embryos from cultured anthers, (2) plant regeneration from the calli or embryos, (3) the percentage of green plants, and (4) chromosome doubling either spontaneously or induced by colchicine treatments

(Henry and De Buyser, 1985; Szakács et al., 1988).

The frequencies of green plants regenerated from anther culture vary within and between plant species. For example, approximately 20-90% of the plants produced from anther-cultures of wheat (Andersen et al., 1987; Zhou and Konzak, 1992), 30-95% from barley (Powell, 1988; Kasha et al., 1990), 20-60% from rice (Chen, 1986; Quimio and Zapata, 1990), and 70-80% from triticale (Charmet and Bernard, 1984) were albino. Numerous studies have demonstrated that the percentage of green plants from anther culture in cereal crops is under the control of a complex genetic system involving both additive and non-additive nuclear gene action as well as cytoplasmic factors (Sagi and Barnabas, 1989; Tuvesson et al., 1989; Ekiz and Konzak, 1991a, b, c; Larsen et al., 1991). Expression of these genes is influenced by environmental factors such as the growth conditions of donor plants (Bjørnstad et al., 1989) and cold pretreatment of anthers (Powell, 1988; Konzak and Zhou, 1991).

Lazar et al. (1984) examined the frequency of callus formation and plantlet formation by anther culture in a diallel population produced from five spring wheat inbred lines. They reported that general and specific combining abilities were highly significant for both traits. Reciprocal effects were also highly significant for both traits.

Sesek et al. (1988) studied the frequency of green plant regeneration in seven wheat genotypes and found that the frequency of responding anthers ranged from 4.8% to 15%. On the average, 8.8% of the responding anthers regenerated albino plantlets while the frequency of green plantlets ranged from 13.1% to 40%. On the average, 2.2 plantlets per 100 isolated anthers were produced. The differences between genotypes were also large and ranged from 1.1% to 3.6%.

Tuvesson et al. (1989) studied the inheritance of anther-culture response in wheat by producing reciprocal crosses between six varieties with high capacity for green plant regeneration and two with low capacity. Pronounced genotypic effect on embryo and green plant formation was observed, while smaller genetic effects were indicated for regeneration. The authors reported that nuclear genes could explain almost all of the genotypic effect in the material studied. Also, embryo formation

showed heterosis over the high capacity parent for 5 of the 12 hybrids, while percentages of green plant formation from the hybrids were intermediate to the parents. General combining ability could explain 78.8% of the variation for embryo formation among the hybrids, whereas the differences in percentage of green plants were dominated by specific combining ability, accounting for 67.9% of hybrid variation. In addition, a positive correlation was observed between the genetic capacity for regeneration and green plant formation. It was suggested that two different classes of genes affected green plant formation in wheat anther-culture: one class with mainly additive effects modifying green plant percentages through an effect on regeneration percentage, and another class of two or more major genes affecting the original frequency of potentially green structure.

Ziegler et al. (1990) investigated the influence of light on regeneration of green versus albino plants from anther culture of wheat. They reported that regeneration under different light conditions resulted in a significantly larger proportion of green plants than in the dark. When embryoids were transferred into the light for differentiation, about 94% of the regenerated plantlets were albino. The embryoids that differentiated in the dark received light only occasionally, when the plates were checked for growth. This short exposure to light seemed to be enough for chlorophyll development; only 73% of the regenerants were albino.

Kao et al. (1991) investigated the effect of culture conditions on induction of green plants from barley microspores during anther-culture. They obtained the highest frequency of plant formation when the anthers were cultured in the dark on a high ficoll medium containing 2,4-D and kinetin for induction of preembryos or callus formation. Subsequently, the proembryos or calli were cultured in dim light on a high ficoll-high sugar medium containing IBA and kinetin and later the embryos were transferred to a starch agar medium. They reported that the ratios of green to albino microspore-derived plants varied from 9:1 to 1:9 depending on culture conditions. They hypothesized that under anaerobic conditions, lactic acid and other organic acids may have damaged the organelles in the cells resulting in the formation of albino plants. Thus, as stated earlier, for direct embryogenesis, using a well-buffered, high ficoll-high sugar medium and proper aeration are essential for

obtaining high frequency of green plants from microspores.

Jahne and Lörz (1995) reported that albinism is a major problem encountered in cereal anther and microspore culture. Many factors have been found to affect the degree of albinism, such as the genotype and physiological state of the donor plants, the developmental stage of the microspores, culture temperature and cold pretreatment. Previously it has been shown that albino pollen plants of barley do not contain mature chloroplasts (Clapham, 1973; and Dunford, 1989), and that albino plants of rice lack major plastid gene products such as 23S and 16S rRNAs (Sun et al., 1979). These workers also reported that albino wheat and barley pollen plants contain extensively deleted forms of the plastid genome, and in rice the albino plants regenerated from microspores contained large-scale deletion structures of the plastid genome. However, the primary cause of albinism is not yet understood.

2.2. Ploidy Level of Regenerants

Kudirka et al. (1983) examined the cytogenetic characteristics of wheat plants regenerated from anther calli of 'Centurk'. Chromosome counts and estimates of ploidy level were made on cells from roots of developing plants obtained by anther culture. Plants with polyhaploid cells were regenerated from all calli, indicating that they were of microspore origin. Three populations were recognized: first, those that were polyhaploid and euploid; second, almost totally polyhaploid and aneuploid; and third, those plants which were largely hexaploid with some cells reflecting spontaneous chromosome doubling. The workers also reported aneuploid cells with corresponding polyhaploid and hexaploid chromosome numbers in root-tip cells. Regenerants exhibiting this phenomenon indicate that the cells in question resulted from doubled haploidy and were not hexaploid cells of the anther wall. Mixoploid plants were regenerated from secondary calli in which chromosome doubling was known to have been induced at the callus stage. In addition, the presence of individual mixoploid root tips in these plants was assumed to indicate that individual organs of a plant may arise from several separate cells of a callus.

In a further experiment Kudirka et al. (1989) studied the ploidy levels of 91

anther culture-derived plants of the wheat cv. Centurk. The cells from regenerants were assayed for ploidy and plants were categorized as polyhaploid, mixoploid or hexaploid. Tillering and seed set were analyzed in plants that survived to maturity. Less than 1% of the tiller population produced by polyhaploid plants set seed. In contrast, 73% of the tiller population produced by hexaploid plants set seed with significantly greater seed set per fertile tiller. They suggested that the ploidy composition of root tips of young regenerants reflected that of the reproductive structures of mature regenerants. Common patterns of aneuploidy in hexaploid and hyperploid cells found among roots of individual plants confirmed that doubling of the cell genome occurred before plant regeneration. Polyhaploid and hexaploid cells were found in individual root tips of mixoploid plants regenerated from calli that were known to be cytochimeric.

Wenzel and Foroughi-Wehr (1984) estimated that in the anther culture of the cereals, most of the regenerated plants are diploid, and the rest are either haploid or tetraploid. They also reported that the pollen stage at the time of culture, growth regulators, or certain epigenetic factors affect the induced ploidy levels. Amssa et al. (1980) suggested that endoreduplication and endomitosis in *T. aestivum* pollen were enhanced by cold pretreatment. Chen and Beversdorf (1992) observed a linear relationship between percent of spontaneous diploidization and cooling temperature. Spontaneous doubling in wheat cultures increases with the length of culture time (Xi et al., 1986) and sugar combination in the media (Navarro-Alvarez et al., 1994).

Charmet et al. (1986) investigated the origin of aneuploid plants obtained by anther culture in triticale. Of the 408 androgenetic plants checked for their chromosome numbers, 228 were aneuploid. They suggested that most of the chromosome variations observed probably preexisted in microspores of the F_1 hybrids. These variations could be caused by meiotic irregularities. On the other hand, the majority of these phenomena involve R-genome chromosomes, which also give rise to meiotic univalents. The chromosome number, and frequency distribution of the microspores from a fairly asyndetic hybrid fits well with that of androgenetic plants.

Devaux (1988) compared the efficiency of anther-culture and the *Hordeum*

bulbosum L. method in winter barley. This study included the chromosome number distributions of all plants derived from the two techniques and proportions of fertile DH plants that survived until maturity. The frequency of haploid and spontaneously DH plants, which were useful for practical breeding, was found to be around 90% for both techniques. The remainder consisted of polyploid, mixoploid and aneuploid variants. The ploidy level distributions of the microspore- and *H. bulbosum*-derived plants appeared to be independent of the genotype of the donor. It was also reported that of 147 microspore-derived plants, 41 (27.9%) were haploid, and 93 (63.3%) were spontaneously DHs. In addition, 10 plants (6.8%) were tetraploid, two were aneuploid ($2n = 2x + 1 = 15$) and one was triploid. On the other hand, 191 (90.5%) of 211 *H. bulbosum*-derived plants were haploid. The remaining 20 plants (9.5%) were interspecific hybrids. It was stated that DHs produced spontaneously from microspore culture (>60%) offered substantial advantages in large-scale DH production even without colchicine treatment. The mean number of seeds set from spontaneously doubled plants was found to be higher than in the case of colchicine-treated haploid plants. This phenomenon obviously has implications in breeding.

Jahne and Lörz (1995) discussed the ploidy level of anther culture- and microspore-derived regenerants in cereals. They reported that microspores, when cultured *in vitro*, exhibited the capacity to spontaneously double the gametophytic chromosome number. However, for unknown reasons, this did not occur in all cases. In wheat anther-culture, the reported figures over a range of genotypes varied from 20% to about 50% of the green regenerants being doubled, while in barley spontaneous dihaploid plants were up to 87%, and in rice up to 72%.

2.3. Chromosomal Variations

McComb (1978) reported that in many species haploid-plantlet regeneration is influenced by the parent plant and the culture conditions. The yield of haploids varies in relation to the stage of microspore development at the time of culture. Rashid and Street (1973) noted a relationship between the age of the donor plant, its hormone status and the proportion of haploid plants. Donor plant growth conditions, the precision of microspore meiosis in the mother plant, abnormal arrangements of

tetrads of spores and the occurrence of dimorphic pollen, culture media, and cold pretreatment also affected the chromosomal constitution of the regenerants. There are various ways in which the developing embryoid could become polyploid. Irregular meiosis, endomitosis, spindle fusion, nuclear fusion and endoreduplication are the main phenomena that contribute to polyploidy (McComb, 1978). In callus cultures various chromosomal abnormalities may persist and predominate due to spindle fusion and endoreduplication leading to euploidy. Multipolar mitoses, bridges and lagging chromosomes lead to aneuploidy (Bayliss, 1973).

Chen and Chen (1980) investigated the changes in chromosome number in microspore-derived callus of rice during successive subcultures. They established 46 callus cultures and maintained them on a medium containing 2,4-D. They reported that at the end of the first transfer 24% of the cultures were non-haploids consisting of only diploid or polyploid cells, or of cells of the two ploidy levels. A small proportion of the cells had $2x$, $4x$, and $8x$ chromosomes. Of the 17 cultures studied cytologically through 19 transfers, chromosome numbers in 13 cultures fell into a ploidy series (x, $2x$, $4x$ and $8x$). Since no diplo- and quadruplochromosomes were observed, the authors inferred that endomitosis rather than endoreduplication was responsible for the changes. In spite of a tendency for chromosome doubling, the proportions of cells of different ploidy levels were fixed in the 13 cultures at later transfers. Eventually haploid cells got eliminated from all cultures and in eight of the cultures diploid cells became the predominant type.

McCoy et al. (1982) studied the frequency and types of chromosomal variability in regenerated oat (*Avena sativa* L.) by detailed meiotic analysis on 655 regenerated plants. The tissue cultures were initiated from immature embryos of the varieties Lodi and Tippecanoe and maintained by monthly subcultures. Among the plants regenerated from 4-, 8-, 12-, 16-, and 20-month-old cultures, cytogenetic alterations were common, although 'Lodi' cultures produced a higher frequency of cytogenetically abnormal plants at each regeneration cycle than 'Tippecanoe' cultures. After four months in culture, 49% of 'Lodi' regenerated plants were cytogenetically abnormal, whereas only 12% of 'Tippecanoe' plants were abnormal. The frequency of cytogenetically abnormal plants increased with culture age. After

20 months in culture, 88% regenerated plants of 'Lodi' and 48% of 'Tippecanoe' were cytogenetically abnormal. The workers also reported that the most common cytogenetic alteration was chromosome breakage, followed by a loss of a chromosome segment resulting in a heteromorphic pair at diakinesis. Other alterations included trisomy, monosomy and interchanges.

Karp and Maddock (1984) studied chromosomal variations in wheat plants regenerated from cultured immature embryos; 29% of the 192 plants examined were aneuploid with chromosome numbers ranging from 38-45, the most frequent being 41 and 43 chromosomes. Of the structural changes in chromosomes, interchanges were the most commonly observed; no inversions were found. Karp (1991) reported that *in vitro* plant cell and callus cultures were also subject to chromosome instability. The occurrence of polyploidy, aneuploidy, and structural chromosome rearrangements were influenced by genotypic, nutritional, and other environmental factors.

Xingzhi and Han (1985) examined the chromosome constitution of F_1 hybrids derived from pollen of hexaploid triticale × common wheat. They studied 588 plants derived from 38 donor plants from three F_1 hybrids on four different media. There was no significant difference among the pollen plants from different donor plants for the distribution of chromosome numbers. The authors suggested that chromosomal constitutions of the pollen plants were determined primarily by the chromosome content of the gametes. Pollen plants having more than 34 chromosomes were considered to have spontaneously doubled chromosomes and were classified as homozygous diploids. A few plants having 43, 45, 49, 64 and more than 90 chromosomes were also obtained.

Murata (1989) studied the effects of auxins (NAA, 2,4-D and 2,4,5-T) and a cytokinin (kinetin) on induction of sister chromatid exchanges (SCEs) in cultured cells of wheat. In the MS medium supplemented with 2.0 mgL^{-1} 2,4-D, the mean number of SCEs per cell was 15.2, and 0.42 per pg of DNA. No significant effect was found in the treatments of 0.5-10.0 mgL^{-1}, whereas more than 2.0 mgL^{-1} of 2,4,5-T induced dramatic increases of SCEs. Kinetin itself had no significant effect on SCEs induction, but there was a tendency that SCEs induced by 2,4,5-T were

suppressed by kinetin.

Pijnacker and Ferwerda (1994) investigated the effect of 2,4-D and sucrose on SCEs in cultured immature embryos of *T. aestivum, T. durum, T. dicoccum*, and *T. monococcum* and regenerants of these lines. They reported that media components may affect DNA replication in cell cultures from the start of culture. Either more 2,4-D and/or more sucrose resulted in more SCEs per chromosome in the diploid, tetraploid, as well as hexaploid wheats except in one genotype. How 2,4-D and sucrose produce alterations in DNA replication is not known. The effect of the 2,4-D and sucrose were additive and the exchanges occurred randomly within the chromosomes. The tetraploids, *T. dicoccum* and *T. durum,* had relatively high mean numbers of SCEs and the diploid *T. monococcum* had low means.

Winfield et al. (1995) cytogenetically analyzed 18 cell lines of bread wheat: 9 microspore-derived, 6 anther culture-derived, and 3 immature-embryo derived. They reported that chromosome variation, both structural and numerical, in all lines studied. Within any given culture, the pattern and extent of variation changed throughout the course of the study and cells with euploid constitution generally decreased in frequency with culture age. Among the nine microspore-derived suspensions, morphogenic lines generally showed a more restricted range of chromosome numbers and higher proportions of euploid cells than nonmorphogenic lines. They found that the patterns of distribution of chromosome numbers among the anther-derived cultures were similar to those of the microspore-derived lines, but the correspondence between instability and regenerative capacity was lower. The immature embryo-derived lines, which were neither regenerable nor morphogenic, were all unstable. The workers sampled the anther-derived lines for several months to determine whether loss of morphogenic potential was related to changes in chromosome instability of specific lines. Analysis of the "elite" line F1.7, initially capable of regenerating green plants, showed that substantial decreases in the frequencies of normal euploid cells (from 45% to 5%) occurred over the period when morphogenic capacity was lost. However, whether the chromosome instability resulted in loss of morphogenicity or vice versa was not clarified. C-banding analyses of lines F1.7 and C82d indicated that instability was not random with

respect to the three genomes (A, B, and D) of wheat or to the different chromosomes within the genomes. Chromosomes of the B-genome were most often lost or involved in rearrangements, with breakpoints located at or near the heterochromatic blocks.

Henry et al. (1996) initiated somatic embryogenesis from immature embryo culture of *T. aestivum* stocks including disomic, ditelosomic and nullisomic-tetrasomic CS wheats. They used the chromosome counts of root tip cells and observed tissue culture-induced variation in the plants regenerated after both short- (4 months) and long-term (14 months) culture. They reported that short-term regenerants from the aneuploid genotypes produced on an average more plants (14%) with abnormal chromosome complements than did the euploid CS line (3.5%). They also observed that most of the aneuploid karyotypes arose from an imbalanced chromosome number in the starting immature embryos. Compared with CS, ditelosomic lines such as DT 4BS, DT 6BL and DT 7DL showed a highly significant increase in somatic chromosome number instability after short-term culture. The frequency of regenerated plants with karyotype abnormalities reached 80% after extended culture time, in both the euploid and aneuploid lines, demonstrating that abnormalities were induced during the *in vitro* culture process. They found that after 14 months of culture, DT 1AL, DT 2AL and DT 7BL lines were more stable than CS. The authors suggested that regeneration through somatic embryogenesis did not ensure normality in the chromosome complement. After long-term cultures, the regeneration capacity was unchanged despite the fact that 80% of regenerated plants possessed an abnormal chromosome complement.

Christensen et al. (1997) examined the anther-culture response of orchard grass, *Dactylis glomerata* L., using two types of media and two sugars to compare their effectiveness. They found out that using maltose instead of sucrose increased the embryo formation and plant regeneration. The ploidy level of the regenerants varied; 22 plants were diploid, 29 were tetraploid, and three octoploid. The workers stated that there was a strong indication that the higher ploidies had arisen through spontaneous chromosome doubling during anther-culture.

Stober and Hess (1997) reported that the ploidy level of anther culture-

derived wheat plants varied. They obtained plants at polyhaploid ($n = 3x = 21$), hexaploid ($2n = 6x = 42$), and heteroploid (e.g. nonaploid, $3n = 9x = 63$, dodecaploid, $4n = 12x = 84$, or aneuploid) levels.

2.4. Gametoclonal and Somaclonal Variations

Genetic variability induced in cultured plant cells was first reported Murashige and Nakano (1967). Ahloowalia was among the first to report tissue culture-induced changes in annual and perennial ryegrasses (Ahloowalia, 1976). The variation observed among plants regenerated from cultured gametic cells is termed gametoclonal variation (Evans et al., 1984; Morrison and Evans, 1987). The concept of gametoclonal variation evolved from that of somaclonal variation, a term first proposed and promoted by Larkin and Scowcroft (1981). They suggested "somaclonal variation" as a general term to describe variation among plants regenerated from cultured cells or tissue. This term has since been widely used in plant cell and tissue culture. The mechanism for induction of somaclonal variants in many culture systems suggests that the genome of higher plants undergoes changes, and that the regeneration of plants from single cells effectively captures this variation (Huang, 1996). The concept of somaclonal variation is built on the classical definition of clone as a population of cells or organisms derived from a single cell or common ancestor by mitotic divisions (Webber, 1903, cited by Rieger et al., 1991). Variations can be induced in the mitotic process either during plant development or *in vitro* cell culture. On the other hand, the term "gametoclonal variation" has been used to describe variation among derivatives of gametic cells in culture.

Evans et al. (1984) reported that somaclonal and gametoclonal variation depend on the occurrence and recovery in regenerated plants of mendelian and non-mendelian genetic variation from cell cultures. The genetic variation in these plants appears to result from both preexisting variation in the donor tissue as well as from variation induced by cell culture. These changes in the integrity of the genome are attributed to mutant induction, mitotic cross over, and organelle mutation or sorting. Based on nuclear genetic differences between differentiated cells and genetic

phenomena occurring during the first few mitotic divisions of callus formation, D'Amato (1978) stated that an explant comprised a heterogeneous cell population from as early as the first days of culture. During growth of callus or cell cultures, additional chromosomal changes appeared to occur in high frequency. Variation in chromosome number was apparently influenced by several factors: 1) Preexisting chromosome variation in plants used for culture initiation; 2) nuclear fragmentation associated with the first cell division of callus initiation; 3) endoreduplication or endomitosis occurring during culture initiation; and 4) abnormalities of the mitotic process resulting in aneuploid cells.

Evans (1989) reported that plant regeneration resulted in transient (epigenetic) variation as well as genetic variation. Epigenetic changes were usually induced by tissue culture, but they are not useful for crop improvement because they were not expressed in the sexual progeny of regenerated plants. He further reported that many of the genetic changes that have been detected either in spontaneous mutants or as a result of induced mutations are also detected via somaclonal variation. Polyploidy is the most frequently observed chromosomal abnormality. Aneuploidy has also been frequently observed in cell cultures. In addition to numerical chromosome changes, mitotic abnormalities such as anaphase bridges, fragments and chromosome rearrangements have been reported. Based on the experiments with several crop species, the following variation has been documented based on evaluation of R_0 or R_1 plants: Cytoplasmic gene changes, chromosome rearrangements, mitotic crossing over, gene expression, copy number of repetitive DNA, activation of transposable elements, tissue-specific changes.

Lee and Philips (1988) reported that in cultured cells the predominant type of aberration was the result of changes in chromosome structure. Therefore, events leading to chromosome breakage, and in some instances subsequent exchange or reunion of fragments, appear to be of fundamental importance. Most events result in chromatin loss. The extent of the loss has ranged from nearly complete elimination of chromosome arms (yielding telocentric or acrocentric chromosomes) to deletions of 10-20% of the chromosome arm length. Another significant event results in exchange of large chromosome segments among nonhomologous chromosomes to

produce reciprocal translocations. The possible origins of chromosome rearrangements in tissue culture are due to two mechanisms: the first, based on late replication of heterochromatin; and the second, an outcome of nucleotide pool imbalance.

Any perturbation affecting the synchrony between chromosome replication during the S phase and cell division would likely result in chromosome aberrations. Because heterochromatic regions replicate later than euchromatic segments, their integrity may be particularly vulnerable to fluctuations in the cycle.

Intracellular deoxyribonucleotide (dNTP) pools have an important influence on the fidelity of several components of prokaryotic and eukaryotic DNA metabolism, including precursor, biosynthesis, replication, repair, recombination, and possibly degradation (Haynes, 1985). Imbalances in the dNTP pools may have serious genetic consequences; these include nuclear chloroplast and mitochondrial mutation, mitotic recombination, chromosome (structural) aberrations, aneuploidy, SCEs, increased sensitivity to mutagens, and oncogenic transformation (Kunz, 1982).

The value of variant plants regenerated from cell culture was first recognized by researchers working with sugarcane (Heinz and Mee, 1969). Potential applications of somaclonal variations have been proposed as a supplementary tool to well-established breeding approaches such as cross-breeding and mutation-breeding for crop improvement. A somaclonal variant wheat cv. Hezu 8 has been officially released for commercial production (Gao et al., 1992). However, the frequency and type of somaclonal variations in a number of plant species, particularly in cereal crops, are low and largely undesirable (Cheng et al., 1992).

Hashim et al. (1988) stated that somaclones with desirable characteristics could be used to improve wheat. Moreover, isozymic analyses of wheat somaclones could help identify variants whose traits are not readily expressed at the morphological and cytological levels.

Rode et al. (1985) analyzed the chloroplast and mitochondrial components of the wheat line Moisson and its anther culture-derived DH lines. Comparisons were made on the basis of their DNA restriction patterns. They reported that no noticeable difference could be detected between the DH lines and the parental line at the ctDNA

and mtDNA level. They concluded, therefore, that *in vitro* culture by itself does not systematically generate a cytoplasmic variation in germ cells.

In a further study, Rode et al. (1987) analyzed the organization of the nuclear ribosomal DNA from a parental line of wheat, Cesar, and its anther culture-derived first cycle and second cycle DH lines. They probed restricted DNA with three subclones of wheat nuclear ribosomal DNA covering the entire repeat unit. No significant difference was detected in the extent of methylation of ribosomal DNA of the DH lines with respect to the parental line. However, they found variation in the organization of the nontranscribed spacer region of ribosomal DNA of the first cycle DH lines. They reported that this variation remained stable after a second cycle of *in vitro* androgenesis. They also pointed out that the occurrence of variations in DNA organization in response to *in vitro* androgenesis probably depended on environmental as well as genetic factors.

To determine the level of gametoclonal variation, Baenziger et al. (1989) studied the agronomic performance of wheat doubled-haploid lines derived from anther-culture. They compared the DH lines from cultivars Kitt, and Chris, to single-seed descent-derived lines (SSDLs) of the same cultivars, and to original cultivars for seven agronomic traits. In both cultivars, the DH lines averaged lower grain yields than the SSDLs, though the difference was only significant for 'Kitt'. In addition, there was significant variation among the DH lines and SSDLs for grain yields. Because in the absence of gametoclonal variation the DH lines and SSDLs should have had similar means, ranges, and genetic components of variance for the measured characteristics, the authors inferred that the variation among the DH lines could be caused by the anther-donor plants being heterogeneous and/or by gametoclonal variation.

Marburger and Jauhar (1989) analyzed microspore-derived DHs of wheat cv. Chris for the presence of variation in agronomic characters, isozyme patterns, and meiotic traits. They reported that heading date, height, number of kernels per spike, 1000 kernel weight, and yield per tiller showed significant variability. The DHs were 19% lower in yield than 'Chris'. They also observed variations in isozyme patterns and meiotic abnormalities occurring in plants of selected DH lines that

significantly differed from the parent cultivar in agronomic characters. To determine the chromosomal basis of phenotypic and isozymic variability, they conducted a meiotic study on DH lines that showed the most differences regarding these traits compared to the parent line. The authors observed various types of abnormalities: sticky chromosomes at metaphase I, asynchronous meiosis, heteromorphic bivalents, interchanges as evidenced by quadrivalent formation, trisomy for a full or part of a chromosome, isochromosomes, interchange heterozygosity, aneuploidy disjunctional abnormalities, including laggards and bridges at anaphase I, and micronuclei at telophase I, or two univalents of different sizes, asymmetrical interchange configuration. They suggested that chromosome abnormalities might have been responsible for altered enzyme activity and agronomic performance of the DH lines.

Mohmand and Nabors (1990) examined somaclonal variants of wheat derived from mature embryo explants of three genotypes. They reported that the somaclones and their parents showed significant variations in spike length, number of grains per spike, and 100-grain weight. They concluded that variations observed in R_1 generations was genetic.

Bentolila et al. (1992) studied genetic segregation in DHs derived by anther-culture in maize crosses. They found numerous aberrant ratios for marker segregation in the DH lines with 87 RFLPs. All of them were homozygous for one of the parental RFLP. A conventionally developed F_2 population was used for comparison and only one marker showed an aberrant segregation ratio. This aberration was attributed to an altered parental allelic distribution (i.e., the parents were not homozygous for this marker). Aberrant ratios using genetic markers and anther culture-derived DHs have also been reported in barley (Thompson et al., 1991; Huen et al., 1991).

Huang (1996) reported that the degree and type of variation are influenced by many factors, including genotype and physiological conditions of the donor plants, tissue culture environment, and the interaction of the various factors operating simultaneously in the tissue culture process.

Bozorgipour and Snape (1997) investigated the feasibility of using tissue culture techniques to generate and select herbicide-tolerant cells of susceptible

genotypes of wheat arising from somaclonal variation. They cultured the immature embryos of genetically defined varieties and after callus initiation subcultured onto selective media containing 5 μM, 10 μM and 50 μM concentrations of difenzoquat and atrazine. They reported that plants were regenerated from all the selective media except the one containing the highest herbicide concentration. They tested the regenerants as whole plants for their response to spray application of herbicides. For difenzoquat, variation in response from extreme susceptibility to tolerance was observed. However, genetic characterization by progeny testing tolerant lines revealed that the induced variation was not heritable. They did not obtain any plants tolerant to atrazine.

2.5. Application of Double Haploids

Khush and Virmani (1996) reported that deployment of the DH method in a breeding program saves time because DH lines can be evaluated for yield and other quantitative traits with low heritability much sooner than those derived through the pedigree method. The instant homozygosity obtained from using a DH system increases the efficiency of selection for both qualitative (controlled by major genes) and quantitative (controlled by minor genes) characters. They also pointed out that when anther-culture is applied in mutation breeding, it could offer certain advantages. A mutation treatment can be given at the haploid cell level, encompassing thousands of haploid cells, and the mutants can be screened under given conditions and expression of the variation in regenerated plants immediately detected. Kaul (1986) induced male-sterile mutants among DHs obtained through anther-culture in rice. Doubled haploid breeding using anther-culture has also been deployed in rice hybrids showing strong heterosis to develop inbred lines possessing a yield potential similar to that of hybrids (Zhu et al., 1983).

Another application of DH lines is for use in genome mapping. For applied breeding strategies, it is usually sufficient to detect characteristic RFLPs correlated to phenotypes rather than locating genes directly responsible for a character expressed at the phenotypic level. DH lines are being used for genome mapping of major genes and/or quantitative trait loci (QTL) in barley (Zivy et al., 1992) and rice (Huang et

al., 1994). For QTL analysis, DH lines provide excellent materials for truly replicating the study over environment to assess and exclude the effect of genotype × environment interaction (Khush and Virmani, 1996).

According to Hu (1997) application of haploids in genetic studies and breeding is based on the following genetic characters of haploids. Firstly, homozygous diploids can be obtained in one generation via chromosome doubling of the haploid plants, whereas six or seven generations are required for the production of homozygous diploids via conventional breeding methods. Hence haploids can be used to shorten the breeding cycle, saving time, space and labor. Secondly, recessive characters are easily expressed in haploids because each chromosome is in single dose and hence there is no possibility of a recessive allele being masked by a dominant allele. This is particularly valuable in mutation breeding and mutant genetic research. Thirdly, gametic genotypes are fully expressed at the plant level in haploids. Gametic types are therefore not lost and multitudinous plants provide a wide spectrum for selection in breeding as well as facilitate genetic analysis of recombinant gametes. Fourthly, about 90% of haploids and homozygous diploids are produced via anther-culture, together with 10% of aneuploids in wheat (Hu et al., 1978, 1980, 1985) and 10% in maize (Gu, 1986). Aneuploids may be used in genetic analysis of chromosomal transfer, thus creating new genetic studies and breeding practice.

Baenziger et al. (1984) made a comparison between DH and conventional plant breeding methods for winter wheat which has a longer growing cycle due to its vernalization requirement. They reported that a minimum of two years could be saved using DHs instead of F_5-derived lines.

De Buyser et al. released the first anther culture-derived winter wheat cultivar in 1987. They reported that the cross was made in 1978 and anthers were plated in 1979. After increasing the seed in the greenhouse and in winter nurseries, replicated yield trials began in 1982 and the cultivar was licensed for sale after two years in the national trials in 1985. The authors estimated that DH breeding saved four years when compared to a conventional winter wheat breeding.

Snape (1989) indicated that DH systems have the unique genetic property of

allowing completely homozygous lines to be developed from heterozygous parents in a single generation. In self-pollinating species, such as wheat, barley or rice, this property can be used to increase the efficiency of cultivar development. The author reported that DHs could also be used to obtain pure stocks of a new cultivar, which conventionally is produced from progeny derived from single plants of an advanced generation. In the case of DHs, all stocks are identical and no purification is required other than isolation to avoid outcrossing.

Bozorgipour and Snape (1992) studied the relationship between *in vitro* performance of haploid embryos and the agronomic performance of the derived DH lines field evaluation. They reported a positive correlation between coleoptile height of haploid plantlets in culture and final plant height of their DH progeny lines in the field. They suggested that *in vitro* selection for plant height and other linked quantitative or pleiotropic characters could be carried out at the initial stages of a DH breeding program.

Jahne et al. (1994) conducted research for developing a simple and efficient procedure for the biolistic transformation of barley microspores. They estimated that the biolistic transformation of barley microspores could prove useful for transfer of genes of agronomic interest. Homozygous transgenic plants could be regenerated in a very short time. However, the transformation efficiency was not very high due to the varying regeneration frequencies of the microspores and therefore the use of more reliable microspore preparations are used as the target tissue for particle bombardment. Jauhar and Chibbar (1999) recommended the combined use of the haploidy and transgenic technologies in genetic transformation of wheat. Direct incorporation of cloned genes at the haploid level followed by chromosome doubling would allow stable integration of transgenes.

Pauls (1996) stated that utilizing DH populations for genetic studies offered several advantages that arise from the fact that the genotypes and genetic ratios in these populations are equivalent to those found in the gametes. Compared to commonly used F_2 populations, DH populations have fewer genotypic classes and larger differences among the classes they contain. Also, much smaller DH populations than F_2 populations are required to observe all possible genotypes from a

cross. In addition, when the phenotype of interest is conditioned by recessive alleles, the fact that the DH population does not contain heterozygotes, in which these alleles are masked, is an important advantage.

Moieni et al. (1997b) examined the DH lines derived from anther-culture of two Iranian spring wheat cultivars, Ghods (susceptible to yellow rust) and 9106 (resistant to yellow rust), and their F_1 hybrids. They inoculated the seedlings of 36 DH lines with eight races of yellow rust. The parental genotypes were segregating for some races but their DH lines were either resistant or susceptible. After further investigations under Iranian field conditions it was found that some of these lines could be used as donor genotypes for resistance to yellow rust in wheat breeding programs. They concluded that anther culture provides an efficient method for fixing genes of resistance to yellow rust and that desirable DHs from F_1 plants can be derived.

3. MATERIALS AND METHODS

3.1. Materials

Ten cultivars of durum wheat (*Triticum turgidum* L., $2n = 4x = 28$; AABB) were used as anther donors in this experiment. The origin and source of the cultivars are given in Table 3.1.

Table 3.1. The list of donor plant genotypes.

Designation	Genotype	Developed by	Origin
G-1	Gediz-75	TRDA-ARI Sahil Kuşağı, 1986	Turkey
G-2	Sham-1	ICARDA, 1991	Syria
G-3	Cosmidor	Introduced cultivar	France
G-4	Kunduru-1149	TRDA-ARI Eskişehir, 1967	Turkey
G-5	Ege-88	TRDA-ARI Ege, 1988	Turkey
G-6	Çakmak-79	TRDA-ARI Orta Anadolu, 1979	Turkey
G-7	Diyarbakır-81	TRDA-ARI G.D. Anadolu and Ege, 1987	Turkey
G-8	Fenike	Land race	Turkey
G-9	Dicle-74	TRDA-ARI Sahil Kuşağı, 1979	Turkey
G-10	Kızıltan-93	TRDA-ARI Orta Anadolu, 1993	Turkey

3.2. Methods

3.2.1. Experimental Design

Each experiment was set up with ten genotypes (Table 3.1), three growth conditions (greenhouse, field, and growth chamber), and four induction media (BAC-1, BAD-1, BAD-3, and M-42). The 120 combinations ($10 \times 4 \times 3$) were repeated 36 times, making a total of 4320 replicates. Number of anthers forming calli and/or embryoids (or anther response), calli formation, albino plant production, and green plant regeneration were recorded.

3.2.2. Growth of Donor Plants

3.2.2.1. Greenhouse

Seed of ten durum wheat cultivars was planted weekly in 15-cm plastic pots (two pots per genotype and two seeds per pot) filled with Sunshine Mix No. 1 (Sun Gro Horticulture, Bellevue, WA) in a greenhouse (attached to the Northern Crop Science Lab., Fargo, North Dakota, USA) with 36 automatically timed 400-W sodium vapor lights on a 16 h photoperiod (as a supplementary to natural lighting), and temperature was maintained at 20-23°C. Upon emergence of the coleoptile and initiation of the first leaf, plants demonstrating winter-type characteristics were transferred to a 2-4°C vernalization chamber with a 12 h-photoperiod for 6 weeks. Plants were fertilized once at the two-leaf stage with Sierra-Osmocote General Purpose Plus controlled-release fertilizer (15-9-12 plus minor elements) supplied by Scotts Sierra Horticultural Products, Marysville, OH.

3.2.2.2. Field

Seed from all cultivars except winter types were planted in April 1999 in field plots located in Fargo and Casselton, North Dakota. Plots were maintained by North Dakota State University. Seeds were planted using a tractor and seeder, with 3 m centers, 25 cm row spacing, and 10 cm seed density. Winter types were planted in 3.8 cm diameter × 20.95 cm deep cones filled with Sunshine Mix No. 1 (one seed per cone) in February; 288 cones were used for each line. The cones were then transferred to a vernalization chamber for six weeks when the coleoptile emerged and plantlets reached the first leaf stage. A total of 144 plants of each cultivar were transplanted by hand to each of the two field locations in mid-April.

3.2.2.3. Growth Chamber

Seed of all cultivars (Table 3.1) was planted weekly in the greenhouse by the method given above; upon emergence of the coleoptile they were transferred to a growth chamber (8 cool white and 7 warm white fluorescent lights 160-W each, 10 incandescent lights 40-W each, 16 h photoperiod with 60% relative humidity, and

temperature was maintained at 21°C). Cultivars exhibiting winter-type characteristics were transferred to a vernalization chamber by the method described above. After six weeks of vernalization, plants were transferred to a growth chamber. They were fertilized once at the two-leaf stage with the same fertilizer as used in the greenhouse.

3.2.3. Media

Four induction media BAC-1 (Marsolais and Kasha, 1985), BAD-1, BAD-3 (Trottier et al., 1993), and M-42 (Kao et al., 1991) [after modification (2 gL^{-1} fructose, 4 gL^{-1} glucose, 50 gL^{-1} sucrose and 130 gL^{-1} ficoll) (Table 3.2)] were used. The BAD-1 medium was modified for differentiation by using agar in place of ficoll, and MS (Murashige and Skoog, 1962) medium was modified [2 mgL^{-1} kinetin, 1 mgL^{-1} indole acetic acid (IAA), and 30 gL^{-1} maltose replacing sucrose] for plantlet regeneration (Table 3.2).

3.2.3.1. Preparation of Media

Since the amounts of some components were too small to be measured accurately in a liter of medium, stock solutions were prepared for BAC-1, BAD-1, BAD-3, M-42, and MS media. Stock solutions not only facilitate long-term storage at -20°C, but also enable rapid, consistently accurate preparation of media.

1) Preparation of Macro and Micro Minerals Stock-Solutions

Macro-nutrient stock solutions (10 ×) were prepared individually for all media. Ten times the amount of macro components for one liter were weighed precisely, using an analytical balance. Components were first dissolved in 500 ml double-distilled water and the volume brought to 1000 ml. The stock solutions were aliquoted into freezer media bags (100 ml per bag), labeled, and stored in a –20°C freezer.

Micro-nutrient stock solutions (100 ×) were prepared individually for all media. The amount of the micro components for one liter was multiplied by fifty

and weighed on an analytical balance. Components were dissolved in 250 ml double-distilled water and final volume brought to 500 ml. The solutions were aliquoted into 15 ml tubes (10 ml in each tube), labeled, and stored in a –20°C freezer.

2) Preparation of $FeSO_4$ + Na-EDTA Stock-Solutions

Stock solutions (100 ×) of FeSO4 + Na-EDTA were made and used for all media. 3.726 g of Na-EDTA was dissolved in 500 ml double-distilled water and just brought to a boil. The solution was boiled for one minute and 2.78 g ferrous sulfate added to it and boiled for one additional minute. When the solution cooled down to room temperature, the final volume was brought to 1000 ml by adding double-distilled water. The solution was kept in a pyrex bottle covered with aluminum foil, labeled, and stored at room temperature.

3) Preparation of Vitamin Stock-Solutions

Vitamin stock-solutions (100 ×) were prepared individually for all media. Fifty times the amounts of the components for one liter were dissolved in 250 ml double-distilled water and the volume brought to 500 ml. Ten ml of each solution was aliquoted into 15 ml tubes, labeled, and stored in a –20°C freezer.

4) Preparation of Amino Acids Stock-Solutions

Amino acids stock-solutions (100 ×) were prepared for all media except BAC-1. The amounts of the components for one liter were multiplied by fifty and dissolved in 250 ml double-distilled water (for M-42 medium coconut water was also added into the amino acids stock-solutions), and volume brought to 500 ml. Ten ml of each solution was aliquoted into 15 ml tubes, labeled, and stored in a –20°C freezer.

Table 3.2. Media compositions (mgL^{-1}).

Components	BAC-1	BAD-1	BAD-3	M-42	MS
Macro minerals					
Potassium chloride	-	-	70	150	-
Potassium nitrate	1500	1500	1900	2500	1900
Ammonium nitrate	200	200	200	600	1650
Sodium phosphate	150	150	60	75	-
Magnesium sulfate	300	300	300	310	370
Ammonium sulfate	400	400	300	67	-
Potassium phosphate	170	170	300	170	170
Calcium chloride	600	600	450	445	440
Potassium sulfate	-	-	70	-	-
Potassium bicarbonate	-	-	50	50	-
Silver nitrate	-	-	10	-	-
Calcium phosphate	-	-	-	100	-
Sequestrene 330Fe	-	-	-	28	-
Micro minerals					
Potassium iodide	0.8	0.8	0.8	0.75	0.83
Manganese sulfate	5	5	5	10	16.9
Molybdic acid	0.25	0.25	0.25	0.25	0.25
Zinc sulfate	2	2	2	2	8.6
Cobalt chloride	0.025	0.025	0.025	0.025	0.025
Copper sulfate	0.025	0.025	0.025	0.025	0.025
Boric acid	5	5	5	5	6.2
Na-EDTA + $FeSO_4$					
Na-EDTA	37.3	37.3	37.3	-	37.3
Ferrous sulfate	27.8	27.8	27.8	-	27.8
Vitamins					
Citric acid	10	10	5	10	-
Pyruvic acid	10	10	5	-	-
Malic acid	-	-	40	10	-
Fumaric acid	-	-	10	10	-
Succinic acid	-	-	5	-	-
Ketoglutaric acid	-	-	-	5	-
Sodium-pyruvate	-	-	-	5	-
Pyridoxine HCl	0.5	0.5	0.5	1	0.5
Ascorbic acid	1	1	1	1	-
Nicotinic acid	0.5	0.5	0.5	1	0.5
Thiamine HCl	1	1	1	2	0.1
Pantothenate-calcium	-	-	0.1	0.5	-
Riboflavin	-	-	0.02	-	-
Folic acid	-	-	0.02	0.2	-
d-Biotin	-	-	0.02	0.005	-
Choline chloride	-	-	-	0.5	-

Myo-inositol	1000	1000	2000	100	100
Growth regulators					
2,4-D	8	8	4	2	-
2-IP	-	-	0.0001	-	-
Kinetin	-	-	-	0.5	2
Indole acetic acid	-	-	-	-	1
Amino acids					
Asparagine	-	60	160	-	-
Arginine	-	30	80	-	-
y-Aminobutyric acid	-	80	213	-	-
p-Aminobenzoic acid	-	-	-	0.01	-
Serine	-	55	147	-	-
Alanine	-	30	80	-	-
Casein hydrolysate	-	300	53	250	-
Cysteine	-	10	27	-	-
Leucine	-	10	27	-	-
Isoleucine	-	10	27	-	-
Proline	-	10	27	-	-
Lysine	-	10	27	-	-
Phenylalanine	-	5	13	-	-
Tryptophane	-	5	13	-	-
Methionine	-	5	13	-	-
Valine	-	5	13	-	-
Glycine	-	2.5	7	-	2
Histidine	-	2.5	7	-	-
Threonine	-	2.5	7	-	-
Glutamine	-	975	1800	100	-
Carbon sources					
Sucrose	60000	60000	60000	50000	-
Glucose	17500	17500	15500	4000	-
Fructose	-	-	2500	2000	-
Maltose	-	-	-	-	30000
Ficoll	100000	100000	125000	130000	-
Xylose	-	-	-	250	-
Agar (purified)	-	-	-	-	8000
Other add ons					
Coconut water	-	-	-	10ml	-
pH	5.8	5.8	5.8	5.8	5.8

5) Preparation of Plant Growth Regulators Stock-Solutions

Stock-solutions of plant growth regulators for all media were prepared individually: One hundred times the amounts of growth regulators for one liter. 2,4-D was dissolved in a few drops of 50 % ethyl alcohol (EtOH) while kinetin, 2IP, and IAA were dissolved in a few drops of 1N NaOH. An appropriate amount of growth regulator was mixed in 75 ml of double-distilled water and final volume brought to 100 ml. One ml of each solution was aliquoted into 2 ml tubes, labeled, and stored in a –20°C freezer.

3.2.3.2. Making Media with Stock Solutions

One liter of a medium was made when needed. Stock solutions of macro minerals (100 ml), micro minerals (10 ml), vitamins (10 ml), amino acids (10 ml) and growth regulators (1 ml) were removed from the freezer and thawed at room temperature. For BAC-1, BAD-1 or BAD-3 media, all of the stock solutions were combined in a beaker on a stirring-hot plate. 10 ml of Na-EDTA + $FeSO_4$ was added to final solutions. Sucrose, glucose and fructose were added according to individual media recipes given in Table 3.2 and final volume brought to1000 ml by adding double distilled water. After adjusting pH of the media to 5.8 using 1M KOH or 1M HCl, ficoll was added. The final media were heated on stirring-hot plates to dissolve the ficoll; care was taken not to boil the media. After the ficoll was completely dissolved, each medium was filter-sterilized using 0.22µm cellulose acetate membrane bottle-top filters (Corning Costar Corning, NY) under vacuum into an autoclaved glass pyrex bottle in a sterile hood. Media were stored at room temperature.

M-42 medium was made by combining all of the stock solutions except vitamins in a beaker. Na-EDTA + $FeSO_4$ and sugars were added according to the media recipe as given in Table 3.2. Final volume of the solutions was brought to 990 ml and pH was adjusted to 5.8 by the method described above. Ficoll was added and the medium was autoclaved in a glass pyrex bottle at 121°C and 1 kg/cm^2 pressure for 20 minutes. When the temperature of the solution dropped to ~60-70°C, the

vitamin stock solution was added after filter-sterilizing using syringe filters (0.2 μm low protein binding filter, Pall Corporation, Ann Arbor, MI) in a sterile hood. The media were stored at room temperature in a sterile hood.

BAD-1 medium was modified for differentiation of calli and/or embryoids. All of the stock solutions [macro (100 ml) and micro minerals (10 ml), Na-EDTA + $FeSO_4$ (10 ml), amino acids (10 ml), and vitamins (10 ml)] except growth regulators were combined in a beaker. Thirty g of sucrose and 17.5 g of glucose were then added to the solution. Final volume was brought to 999 ml with double-distilled water and pH was adjusted to 5.8 as described above. Eight g of agar (replacing the ficoll) was added into solution and autoclaved in a glass pyrex bottle at 121°C and 1 kg/cm^2 pressure for 20 min. After autoclaving, the bottle was placed on a hot stir-plate in a sterile hood. When the solution cooled down to ~60-70°C, stock solutions of growth regulators (1 ml), after filter-sterilizing using syringe filters, were added into the media. 10 ml of the medium was pipetted into 10 × 60 mm petri plate. The plates were labeled, wrapped with plastic saran wrap and kept in a 4°C refrigerator.

MS medium was modified to use as regeneration medium (Table 3.2). Stock solutions of macro minerals (100 ml), micro minerals (10 ml), growth regulators (1 ml), and Na-EDTA + FeSO4 (10 ml) were combined in a beaker. Thirty g of maltose was added to the solution and total volume was brought to 990 ml and pH was adjusted to 5.8. After pH adjustment, 8 g agar was added to the solutions and autoclaved as described earlier. After autoclaving, the bottle was placed on a hot stir-plate in a sterile hood. When the solution cooled down to ~60-70°C, stock solutions of vitamins (10 ml), after filter sterilization using a syringe filter, were added into the media. 25 ml of the medium was pipetted into each 20 × 100 mm petri plate. The plates were labeled, wrapped with plastic saran wrap and kept in a 4°C refrigerator.

3.2.4. Plant Preparation

3.2.4.1. Collecting Spikes and Cold Treatment

Plants were observed daily (every other day for field plants) and immature spikes were collected when the awns first emerged from the flag leaf. Spikes were removed from the plant by cutting the stem ~1 cm below the first node under the spike, immediately placed in water and brought to the laboratory. All leaves except the flag leaf were then removed and the spike with flag leaf placed in a beaker containing enough distilled water to cover the stem at or just below the node below the spike. The beaker and spikes were covered with plastic wrap, labeled, and refrigerated at 4°C for 7 days.

3.2.4.2. Sterilization of the Spikes

After cold treatment, spikes were removed from the boot (leaf sheath of flag leaf), stems excised 1 cm below the first floret, and put in appropriately labeled 50 ml tubes. The spikes were sterilized with 70% ethyl alcohol (EtOH) for 1 minute, immediately followed by a solution of 1% sodium hypochlorite + 0.1% Tween 20 for 8 min., and then rinsed with sterile double distilled water 4-5 times. After the last rinse a small amount of water (~2 – 4 ml) was left in the tubes to prevent the spikes from drying.

3.2.4.3. Staging Anthers

Before culture, each spike was staged by removing the central anther from the outer floret of the spikelet in the middle of the spike. The anther was macerated in a drop of 1% acetocarmine on a glass slide until immature pollen or microspores were removed from the anther and suspended in the stain. The preparation was then covered with a glass cover-slip and pressed gently between bibulous paper to blot off any excess stain. The preparation was then viewed under a microscope with 10 ×, 20 ×, or 40 × objective and 20 × eyepiece depending on the resolution required to determine the stage of pollen development. Anthers at the mid-uninucleate stage were selected and cultured.

3.2.5. Culturing Techniques

3.2.5.1. Anther Culture

Under aseptic conditions in a laminar flow hood, the florets of the sterilized spikes were gently opened and anthers excised using forceps and scalpels that had been alcohol-sterilized and flamed. When possible, filaments were removed without damaging the anther. Each individually excised anther was placed into a well of a six-well petri plate (flat bottom, 9.6 cm^2, 17.3 ml volume per well) containing 5 ml of induction medium. Twenty anthers were cultured per well. Anthers were placed onto the media so that each hemisphere (lobe) was in contact with the media. This was repeated until all the wells of a plate were used. The culture plate was then covered, sealed with parafilm, appropriately labeled, and transferred to a dark incubator at 28°C.

3.2.5.2. Embryoid and Callus Initiation

Culture plates were observed weekly for calli and/or embryoid development. Anther response and number of calli and/or embryoids were recorded for each well in a petri plate.

3.2.5.3. Albino and Green Plantlets Regeneration

Calli and/or embryoids were removed from induction media when they were approximately 3-4 mm long, and transferred to a differentiation medium (modified BAD-1 mentioned above). Transferred cultures were kept under induction conditions for one week, then moved to an incubation room, at 25°C, and 16 h photoperiod. Cultures were observed periodically and plantlet initiation recorded for each petri plate.

3.2.5.4. Development of the Plantlets

After plantlet regeneration, the cultures were transferred to 25 ml modified MS medium (Table 3.2) and kept in an incubation room (25°C, 16 h photoperiod). Plantlets were transferred to peat pellets and hardened in a growth room. After one week they were moved to the greenhouse, transplanted into pots and grown to maturity.

3.2.6. Cytological Study

3.2.6.1. Chromosome Preparation

1) Somatic Chromosomes

Root tips were excised in the morning at about 9 o'clock and kept in chilled distilled water at 4°C for 24 h. Then, they were fixed in acetic alcohol; 95% EtOH and glacial acetic acid (3:1 by volume). Mitotic chromosome spreads were prepared within two weeks from the time of root-tip fixation. Root tips were squashed on a glass slide in a drop of 45% acetic acid, covered with a glass cover slip and viewed under a phase contrast microscope. Chromosome numbers were recorded. Slides containing well spread metaphase chromosomes were transferred to a –80°C freezer and kept for up to one month (if necessary) for fluorescent genomic *in situ* hybridization (GISH).

2) Meiotic Chromosomes

Immature spikes at the appropriate stage were fixed in the morning (at about 9 am) in Carnoy's fluid containing 95% EtOH, chloroform, glacial acetic acid (6:3:1). Meiotic chromosome spreads were prepared by squashing anthers in 45% acetic acid 3 to 30 days after fixation. The techniques we standardized earlier were followed (Jauhar et al., 2000). Only slides with pollen mother cells (PMCs) containing good metaphase I spreads were scored for chromosome pairing and then frozen at –80°C for up to one month for fluorescent GISH.

3.2.6.2. Fluorescent GISH

1) Probe and Blocking DNA Preparation

Fluorescent GISH was carried out by probing the A genome with *T. urartu* total genomic DNA after blocking the B genome with *Ae. speltoides* total genomic DNA. High molecular weight DNA was extracted from young actively growing *T. urartu* and *Ae. speltoides* leaves by the CTAB method (Doyle and Doyle, 1990).

a) A-Genome Probe

T. urartu genomic DNA was sheared by repeatedly passing it through a 25 Ga. Needle (400–500 times). DNA fragment size was determined by electrophoresis on a 2% agarose gel using the appropriate molecular weight markers. Shearing was considered complete once the DNA bands in the gel were within the 300–600 bp range. The sheared DNA was then labeled with biotin-14-dATP using a BioNick Labeling System (Gibco-BRL) following the protocol supplied with the kit. Activity of the nick-translated probe was determined by a Dot Blot assay (Gustafson et al., 1990), and samples which showed good activity were combined. DNA concentration was reconfirmed by using a spectrophotometer. The probe was then aliquoted, with the required amount of DNA to do four slides, into 0.5 ml tubes and stored at -20°C.

b) B-Genome Blocker

Ae. speltoides genomic DNA was autoclaved for 15 min. at 121°C. The autoclaved DNA was then ethanol-precipitated and resuspended in TE buffer at pH 8.0. Concentration was determined by a spectrophotometer, while fragment size was determined by electrophoresis on a 2% agarose gel and appropriate molecular weight markers. The blocking DNA was then stored at -20°C until use.

2) Hybridization

After storage in a -80°C freezer, cover-slips were removed from a set of four slides using compressed CO_2 gas. The slides were passed through an ethanol dehydration series: 70%, 95% and 100%, at -20°C for 2-3 min. each. The slides were then left to dry. After drying, the slides were transferred to a 60 ml Coplin jar containing 50 ml 2 × SSC and 150 µl (10 mg/ml) RNase A at 37°C for 1 h. Following the RNase A treatment, the slides were washed twice in 2 × SSC at 37°C. The DNA on the slides was then denatured by immersing the slides in a 70% formamide/2 × SSC mixture at 70°C for 4.5 min. for mitotic preparations or 3 min. for meiotic preparations. Immediately after the 70% formamide/2 × SSC denaturation the slides were quenched in 70% ethanol at -20°C for 2 min., and transferred to -20°C 95% and 100% ethanol consecutively for 2 min. each.

The slides were then removed and dried. A hybridization mixture for four slides consisted of 50 µl formamide, 20 µl 50% dextran sulfate, 10 µl 20 × SSC, 20 µl TE buffer pH 8.0 containing 400 ng biotin-14-dATP-labled probe DNA and 2000 ng blocking DNA, and was prepared in a 1.5 ml tube. The hybridization mixture was denatured in a dry-block incubator for 10 min. at 90°C. Immediately after denaturing, the hybridization mixture was quenched on ice and vortexed for the first 30 sec. to maximize cooling. After leaving the hybridization mixture on ice for 5 min., 25 µl was applied to each slide. A plastic cover slip was placed over the hybridization mixture and sealed with rubber cement. The slides were placed in a humidity chamber, moistened with 2 × SSC, and incubated in a forced air oven for 10 min. at 80°C. The slides were then removed and left for ~2 min. to cool and then transferred to a 37°C incubator for overnight hybridization.

3) Probe Detection

The next day, the cover-slips were gently removed and the slides were washed twice in 2 × SSC for 5 min. each at 40°C. The slides were transferred to a 50% formamide/2 × SSC mixture for 10 min. at 40°C, followed by two more 2 × SSC washes for 5 min. each at 40°C. After the final wash, the bottle was then left to

cool to room temperature. The slides were then transferred to a 4 × SSC/0.2% Tween-20 buffer solution for 5 min. at room temperature. To avoid nonspecific binding of the avidin, 200 µl of 5% BSA (Bovine Serum Albumen) in 4 × SSC/0.2% Tween-20 was applied to each slide, covered with a plastic cover-slip, and placed in a humidity chamber at room temperature for 10 min. The cover slip was then gently removed and the excess buffer drained from the slide. 70 µl of fluorescien isothiocyanate (FITC)-conjugated avidin DCS (10 μgml^{-1}) in 5% BSA/4 × SSC/0.2% Tween-20 was added to each slide, covered with a plastic cover slip and incubated for 45 min. at 37°C in a humidity chamber. After incubation, the cover slip was gently removed and the slides washed three times in 4 × SSC/0.2% Tween-20 at 37°C for 5 min. each.

4) Counter Staining

The chromosome preparations were counterstained with propidium iodide (PI) or 4',6-diamidino-2-phenylindole (DAPI). The slides were drained and placed in a humidity chamber, 150 µl of PI (20 μgmL^{-1}) in 2 × SSC or 150 µl of DAPI in 4 × SSC (20 μgmL^{-1}) was added to each slide and covered with a plastic cover slip. The humidity chamber was then incubated in the dark at room temperature for 20 min. After the incubation period the cover slip was gently removed and the slides washed briefly in 2 × SSC for PI is used and 4 × SSC for DAPI. One drop of antifade solution was added to each slide after draining any excess, and covered with a good quality UV-transparent glass cover-slip. The slides were stored in a refrigerator in the dark at 4°C until viewing the next day.

5) Visualizing and Imaging

Slides were observed under a Zeiss Axioskop epi-fluorescent microscope with a Zeiss AttoArc 100 watt adjustable UV light source. To visualize the FITC signal of the labeled probe a Zeiss set code 09 excitation filter with a Chroma D535/40m (Chroma Technology Corp., Brattleboro,VT, USA) emission filter was used. For the PI or DAPI counterstain, a Zeiss set code 00 or set code 02 excitation

filter with a Chroma D 605/55m or Chroma D 460/50m emission filter, respectively, was used. Images were captured at 1315 × 1033, 36 bits/pixel resolution with a SPOT II digital camera (Diagnostic Instruments, Inc., Sterling Hts., MI, USA) connected to a PC using the camera software provided. Images were oriented to the proper layout using Paint Shop Pro v. 5 (JASC Software, 1998), and printed with an Epson Stylus Photo 875DC color ink jet printer at 1440 × 720 resolution.

3.2.7. Data Analyses

Analysis of variance (ANOVA) and mean separation were conducted using SAS computer software (SAS, 1998). The data were analyzed as a 10 × 4 × 3 random effects factorial design with genotype, growth condition, and media as independent effects. Dependent effects were the ratio of anthers forming calli and/or embryoids (= anther response), number of calli per anther, and number of regenerants per anther from individual plates. The error mean square from the ANOVA was used to calculate the LSD for each comparison. The ratio of responding anthers, the number of calli per anther, and the number of regenerants per anther were compared across genotypes, donor-plant growth conditions, and media. Interactions were also studied.

4. RESULTS

4.1. Growth of Parental Cultivars

Phototropic effects in all cultivars (cvs.) were evident under all conditions. This was expected as there is usually some form of growth behavior difference when plants are taken from one region (in this case Turkey) and grown in another region, i.e., North Dakota (ND), USA. Overall plants tended to be shorter than normal when grown in ND compared to their original habitat in Turkey. All cultivars set seed in all primary, secondary, and, in most cases, the tertiary florets, indicating that the fertility was not affected by the change in growth conditions. It was observed, however, that the seed harvested tended to be little smaller than the original seed under all three conditions.

4.1.1. Greenhouse

Parental cvs. responded well under greenhouse conditions, were lush green with good tillering (Figures 4.1 A and B). However, they were typically shorter than when grown under the conditions experienced in Turkey. The parental cvs. developed large, firm spikes and had complete fertility. The cultivars that showed the winter-type growth exhibited, on vernalization, the growth pattern typical of ND winter types. Plants of the same cultivar had uniform growth pattern.

4.1.2. Field

Plants that were planted in the field demonstrated stronger phototropic effects than those grown in the greenhouse or growth chamber. They were semi-dwarf to dwarf (Figure 4.2), matured more rapidly, and were harvested 14 to 16 weeks after planting. The plants that exhibited the spring growth-habit showed good tillering with as many as 15-20 tillers per plant. However, the ones that exhibited the winter-type characteristics did not tiller as well, with only 3-8 tillers per plant. The winter types did not have as much biomass as the spring types; in many cases, the plants consisted of only 3 stems and 9 leaves, and the leaves tended to be smaller

Figure 4.1. Durum wheat cultivars grown under greenhouse conditions. **A.** Cultivar G-7 plant at the proper stage to collect spikes for staging for anther culture. Arrows indicate spikes, which were collected. **B.** Another G-7 plant at the appropriate stage to collect spikes. Note uniformity between the plants in A and B.

Figure 4.2. Durum wheat in the field at Fargo, North Dakota. Note the uniformity of each row (1, 2, 3, and 4). Also note the height of the durum cultivars in rows 1 to 3, compared to the local check Langdon (L).

than those of the same cv. grown in the greenhouse. Spikes from the field plants were not as robust as those from the greenhouse. Plants exhibiting both spring and winter characteristics were fertile, with less than 1% of the primary and secondary florets not filled. Rows of the same cultivar were uniform with one another and no off types were observed (Figure 4.2).

4.1.3. Growth Chamber

Plants did not grow very well under growth chamber conditions. They were shorter than greenhouse- and field-grown plants. Spikes tended to be smaller, and leaves narrower. Overall, the plants looked weak and chlorotic.

4.2. Media

Preliminary work was done on ND lines (unpublished information) prior to the research conducted on Turkish lines. It was determined that modifications of M-42 induction, BAD-1 differentiation and MS regeneration media were needed to improve the efficiency of anther culture. In the preliminary study, M-42 was the worst of the four induction media. The high concentration of ficoll (400 gL^{-1}) seemed to block nutrient absorption and desiccated the anthers, thus drying them out in a few days. The addition of fructose (2 gL^{-1}), increasing the sucrose (by 20 gL^{-1}) and glucose (by 3 gL^{-1}) levels of M-42, and reducing ficoll (by 270 gL^{-1}) improved the response of anthers on this medium. Since the viscosity of the BAD-1 differentiation medium was not high enough to keep 3-4 mm calli afloat, the ficoll component was removed and replaced with purified agar (8 gL^{-1}), resulting in a gel rather than a viscous liquid. For adequate plant regeneration, several modifications to the MS media were tried. Different sugar sources and concentrations, and different growth regulators and amino acids were tried. Finally when sucrose was replaced with 30 g of maltose, and 2 mgL^{-1} kinetin, and 1 mgL^{-1} IAA were added to the original MS medium, it proved to be the most successful for generating green plants.

4.3. Anther Culture

All anthers were cultured at the mid-uninucleate stage (Figure 4.3 A). They were oriented in such a way that both hemispheres (lobes) were in contact with the medium (Figure 4.3 B). It was observed that anthers (at mid-uninucleate stage) from field-grown material were larger than those from the other two growth conditions. Generally, anthers from the growth-chamber plants were the smallest. One week after culture the anthers started swelling, and their color faded to light yellow and eventually to white (Figure 4.4 A). The first signs of anther response or calli initiation were detected 7-10 days after culture (Figure 4.4 B).

A total of 86,400 anthers were cultured. The overall anther-culture response is given in Table 4.1.

Table 4.1. Overall anther-culture response of ten durum wheat genotypes.

	Total	% Yield
Number of replications	36	
Number of treatments	120	
Anthers cultured	86400	
Anthers responded	424	0.49
Calli produced	559	0.65
Total plants regenerated	324	0.38
Green plants regenerated	248	0.29
Albino plants regenerated	76	0.09

4.3.1. Callus and Embryoid Initiation

Microspores that responded to culture developed into either embryoids (Figure 4.4 C) or formed calli (Figure 4.4 D), although in some cases both embryoids and calli were derived from the same anther (Figure 4.4 E). Many of the anthers produced only a single callus (Figure 4.4 D), while others produced several calli. In some cases, a complete hemisphere (lobe) of an anther was covered with calli

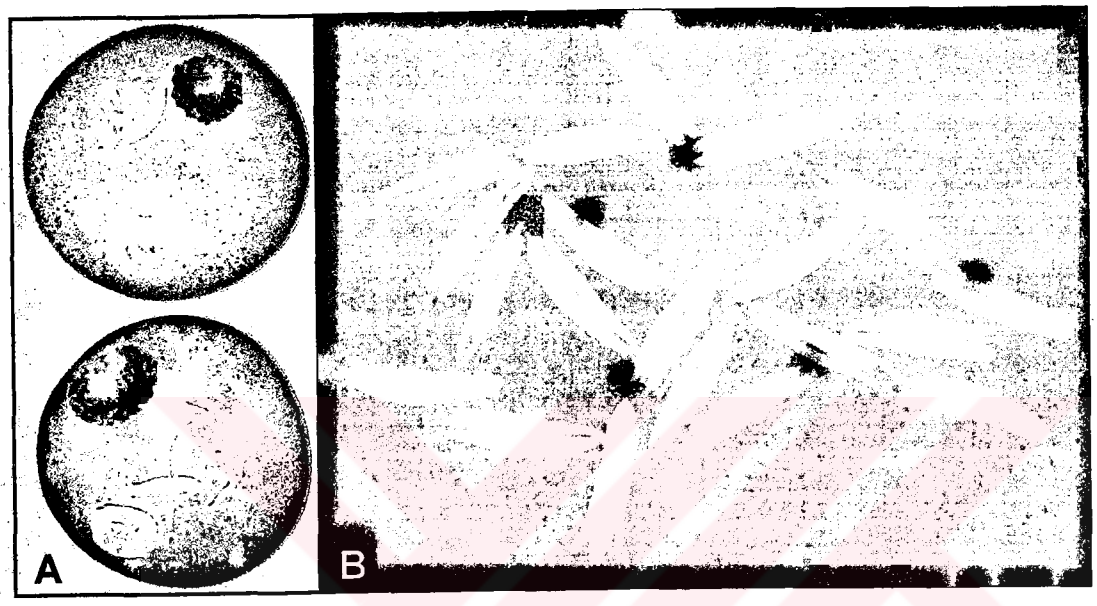


Figure 4.3. Microspores and anthers at the proper culturing stage. **A.** Mid-uninucleate microspores at two separate stages; note the position of nuclei in relation to pollen germpore. **B.** Freshly cultured anthers on liquid medium, with both hemispheres (lobes) in contact with the medium.

Figure 4.4. Stages of anther response and callus induction. **A.** Seven days after culture; note whitish color of the cultured anther. **B.** Callus initiation 10 days after culture. **C.** Embryoid formation from microspore. **D.** Callus formation from microspores. **E.** Anther three weeks after culture showing the formation of both embryoids and calli. **F.** Another anther three weeks after culture, with one hemisphere completely filled with calli while the other hemisphere seemingly collapsed and dried. **G.** Multiple anthers on the induction medium three weeks after culture. Calli were formed primarily in the anther lobe but free floating calli (arrowheads) were also formed from released microspore. Note the formation of filament calli (arrows), which were eliminated during subculture.

(Figure 4.4 F). Free-floating calli were also observed (Figure 4.4 G), as they resulted from microspores after they separated from the dehisced anther. Callusing of the anther filament was common (Figure 4.4 G). Filament callus was removed, whenever found, to avoid its mixing with other calli while subculturing. In some cases, filament calli got detached from anthers during development, but they were easily identified by the cell density and looser cell adhesion.

4.3.1.1. Anther Response

Of a total of 86,400 anthers cultured, a total of 424 anthers responded (0.49%, see Table 4.1). ANOVA results are given in Table 4.2 for anther response of the ten durum wheat genotypes, on four media, and under three growth conditions.

Table 4.2. ANOVA for the effect of genotype, donor plant growth condition, and media on anther response (anthers forming calli/embryoids), calli production, green and albino plant regeneration of durum wheat.

		Anther response		Calli production		Green plants		Albino plants	
Source of variance	***df***	**MS**	**F value**	**MS**	**F value**	**MS**	**F value**	**MS**	**F value**
Model	119	0.85	5.36***	1.66	4.75***	3.43	1.06	0.16	1.07
Error	4200	0.16		0.35		3.25		0.15	
Corrected total	4319								
Genotype	9	2.74	17.26***	5.42	15.51***	9.39	2.89**	0.17	1.13
Growth condition	2	1.72	10.83***	3.62	10.34***	11.22	3.46*	0.74	4.81*
Media	3	4.29	27.09***	8.51	24.33***	1.66	0.51	0.13	0.84
Gen. × Grow. cond.	18	1.22	7.71***	2.25	6.43***	7.44	2.29**	0.19	1.23
Gen. × Media	27	0.50	3.17***	0.98	2.80***	1.93	0.60	0.15	0.99
Grow. cond. × Media	6	1.05	6.62***	2.09	5.97***	1.70	0.52	0.11	0.71
Gen. × Grow. cond. × Media	54	0.34	2.13***	0.68	1.93***	1.84	0.57	0.15	0.95

*** $\rho = 0.0001$
** $\rho = 0.01$
* $\rho = 0.05$
Gen. = Genotype
Grow. cond. = Growth condition

As shown in Table 4.2, the genotype, growth condition, and media had a highly significant effect on anther response. Genotype × growth condition, genotype × media, growth condition × media, and genotype × growth condition × media interactions were also highly significant.

1) Effect of Genotype

All of the ten genotypes showed anther-culture response, although significant genotypic differences were observed. The effect of genotypes on anther response is summarized in Table 4.3. The highest mean anther response was obtained from G-9, while G-6 gave the lowest. G-9 and G-3 were not significantly different from each other, but G-9 was significantly better than the other genotypes.

Table 4.3. The effect of genotypes on anther response (anthers forming calli/embryoids), calli production, green plant and albino plant regeneration.

Genotype	Ratio of responding anthers	Mean no. of calli per anther	Mean no. of green plants per anther	Mean no. of albino plants per anther
G-1	0.0038 cd[α]	0.0045 cd	0.0000 b	0.0002 a
G-2	0.0029 cd	0.0041 cd	0.0000 b	0.0000 a
G-3	0.0101 ab	0.0120 ab	0.0000 b	0.0028 a
G-4	0.0008 d	0.0011 d	0.0000 b	0.0000 a
G-5	0.0039 cd	0.0046 cd	0.0003 b	0.0009 a
G-6	0.0006 d	0.0007 d	0.0000 b	0.0000 a
G-7	0.0068 bc	0.0104 bc	0.0050 ab	0.0003 a
G-8	0.0047 cd	0.0057 bcd	0.0000 b	0.0010 a
G-9	0.0128 a	0.0188 a	0.0234 a	0.0024 a
G-10	0.0024 cd	0.0032 cd	0.0000 b	0.0009 a

[α]Means with the same letter are not significantly different ($\rho = 0.01$)

2) Effect of Growth Conditions

Anthers from cultivars grown under all three conditions responded to culture. The effect of growth conditions on anther response is given in Table 4.4. Anthers from field- and greenhouse-grown materials responded to culture significantly better than those from the growth chamber-grown material. Field-grown material gave somewhat better anther-culture response than the greenhouse-grown material, but they were not significantly different from each other.

Table 4.4. The effect of growth conditions on anther response (anthers forming calli/embryoids), calli production, green plant, and albino plant regeneration.

Growth conditions	Ratio of responding anthers	Mean no. of calli per anther	Mean no. of green plants per anther	Mean no. of albino plants per anther
Field	0.0062 a	0.0082 a	0.0080 a	0.0005 ab
Greenhouse	0.0056 a	0.0077 a	0.0007 b	0.0022 a
Growth chamber	0.0030 b	0.0036 b	0.0000 b	0.0000 b

[a]Means with the same letter are not significantly different ($\rho = 0.01$)

3) Effect of Media

Anther response occurred on all media used in the experiment. The best anther-culture response was obtained on BAD-1, but BAC-1 was not significantly different from BAD-1. M-42 gave the lowest mean anther response but it was not significantly different from BAD-3 (Table 4.5).

Table 4.5. The effect of media on anther response (anthers forming calli/embryoids), calli production, green plant, and albino plant regeneration.

Media	Ratio of responding anthers	Mean no. of calli per anther	Mean no. of green plants per anther	Mean no. of albino plants per anther
BAC-1	0.0064 a	0.0082 ab	0.0000 a	0.0005 a
BAD-1	0.0085 a	0.0118 a	0.0037 a	0.0009 a
BAD-3	0.0034 b	0.0046 bc	0.0034 a	0.0016 a
M-42	0.0013 b	0.0015 c	0.0044 a	0.0005 a

[a]Means with the same letter are not significantly different ($\rho = 0.01$)

4) The Effect of Genotype × Growth Condition Interactions

The effect of genotype × growth condition interactions was highly significant for anther response (Table 4.2). As shown in Chart 4.1, different genotypes responded differently when grown under different conditions. Among the field-grown materials, G-9 gave the highest mean anther response. Under greenhouse conditions, G-8 was the best, whereas under growth-chamber conditions G-7 had the highest mean anther response.

5) The Effect of Genotype × Medium Interactions

The genotype × medium interactions were also highly significant for anther response (Table 4.2). As shown in Chart 4.2, anther response varied for each genotype when they were cultured on different media.

6) The Effect of Growth Condition × Medium Interactions

The effect of growth condition × medium interactions was highly significant (Table 4.2). Chart 4.3 shows that BAD-1 was the most successful media for field grown material. For greenhouse- and growth chamber-grown material, BAC-1 produced a higher anther response than the other media.

Chart 4.1. The effect of genotype and growth condition on anther response

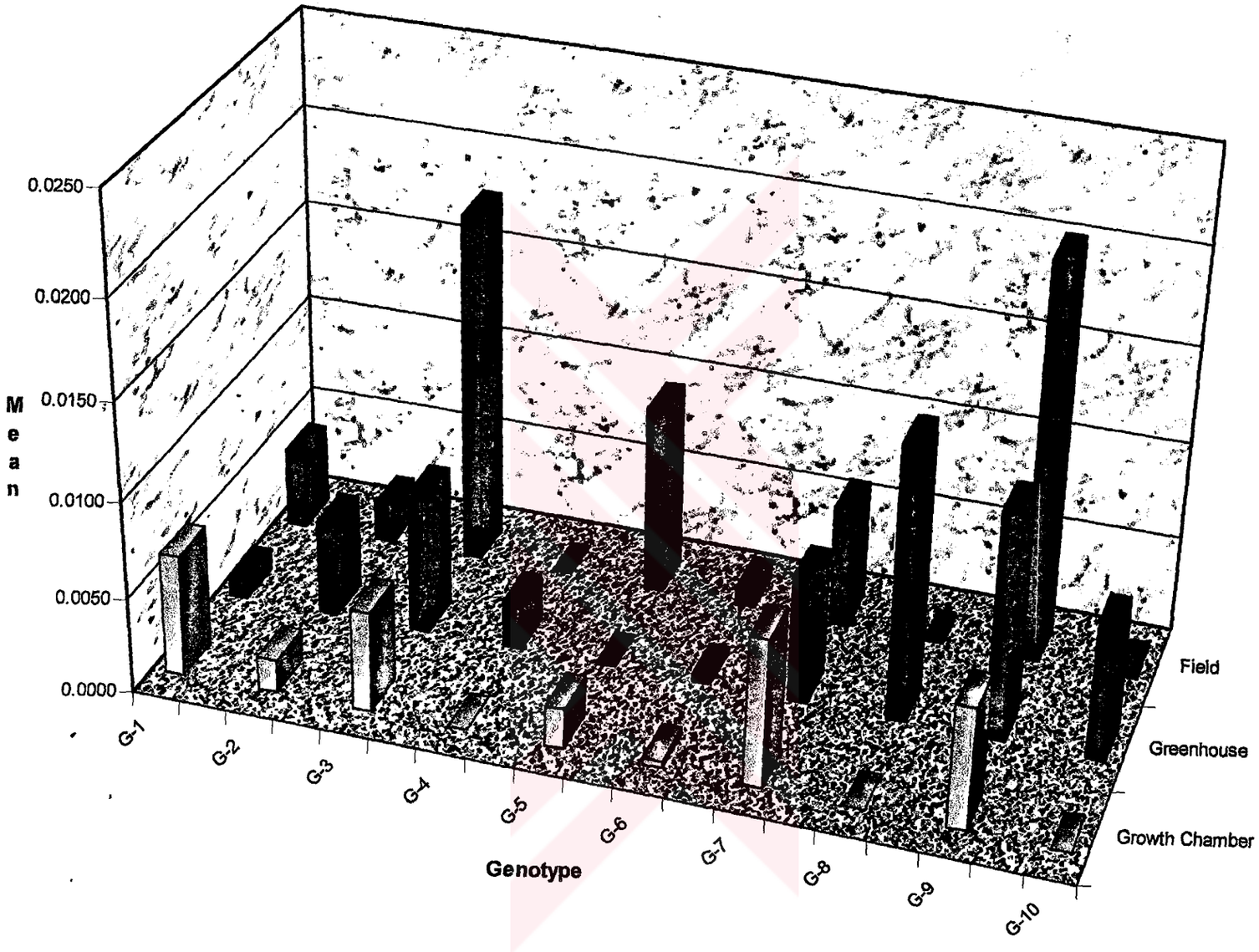


Chart 4.2. The effect of genotype and media on anther response

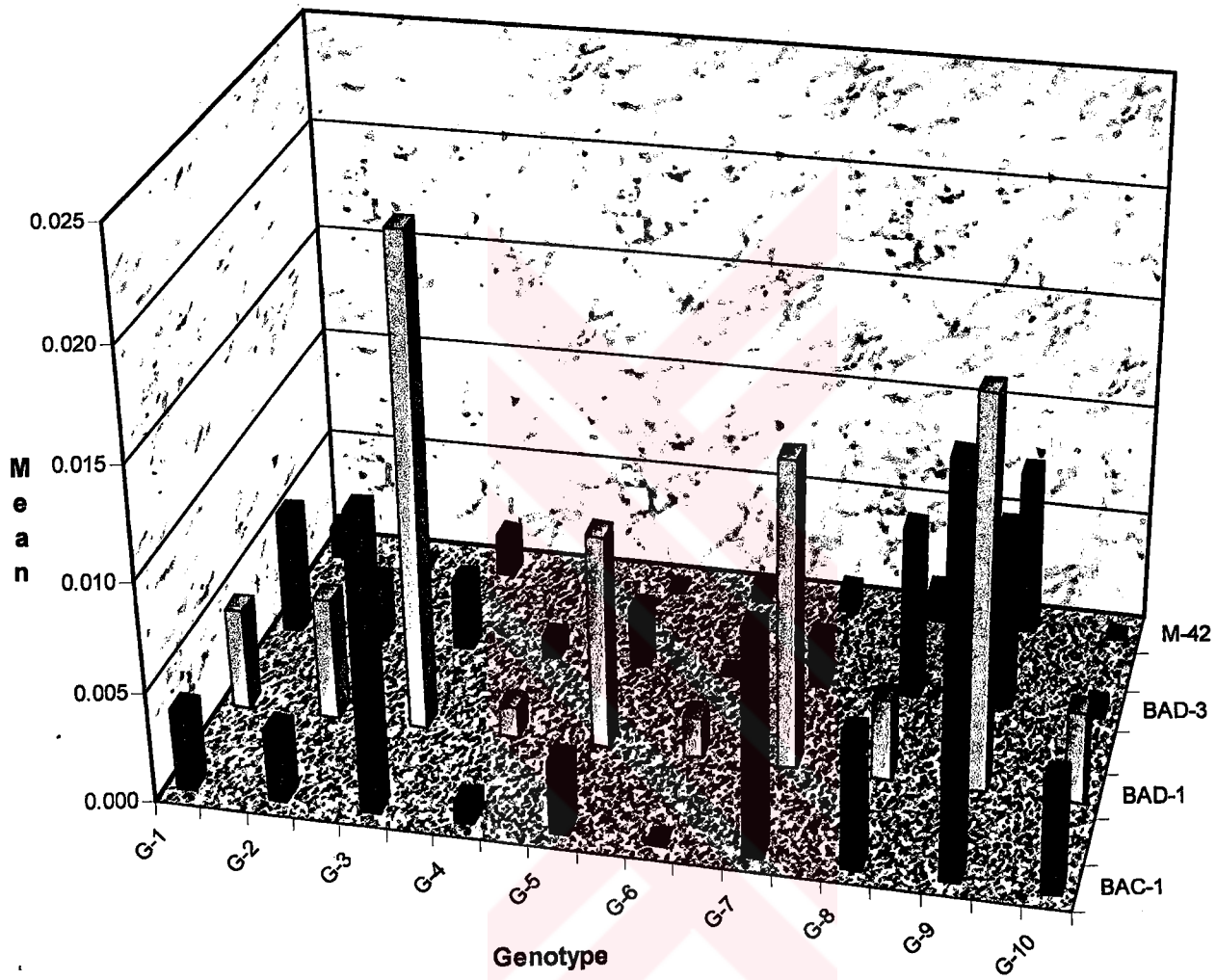
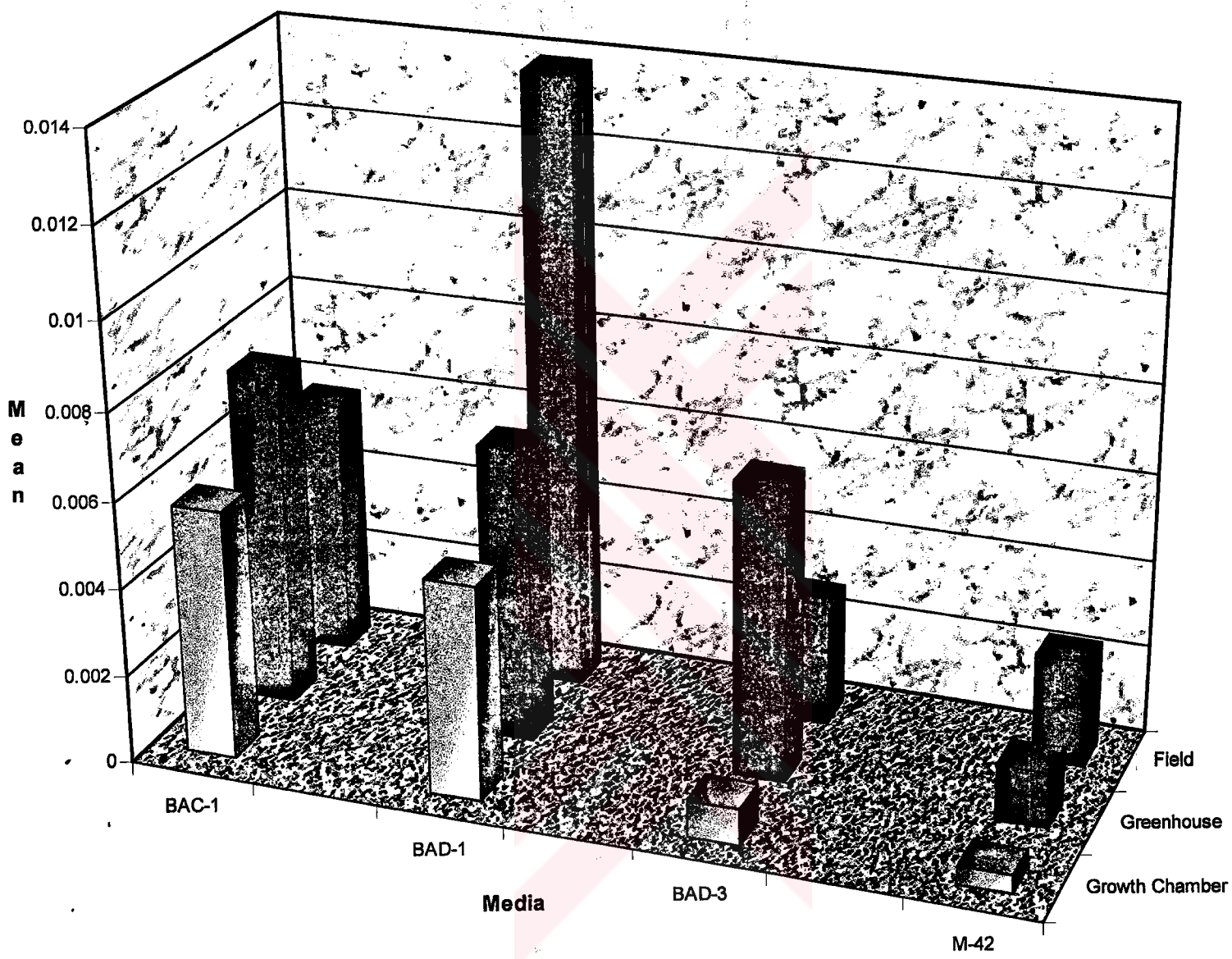


Chart 4.3. The effect of growth condition x media on anther response



7) The Effect of Genotype × Growth Condition × Medium Interactions

Table 4.2 shows that genotype × growth condition × medium interactions were highly significant for anther response. As shown in Chart 4.4, donor plant genotypes from different growth conditions responded differently on the four media. Overall, the highest mean anther response was obtained from field-grown G-3 on BAD-1 medium.

4.3.1.2. Callus Production

From the 424 anthers that responded, 559 calli were obtained (0.65%, Table 4.1). ANOVA results for callus production of the ten durum wheat genotypes, four media, and three growth conditions are given in Table 4.2. Genotype, growth condition, and media strongly affected calli production. Genotype × growth condition, genotype × medium, growth condition × medium, genotype × growth condition × medium interactions were also highly significant.

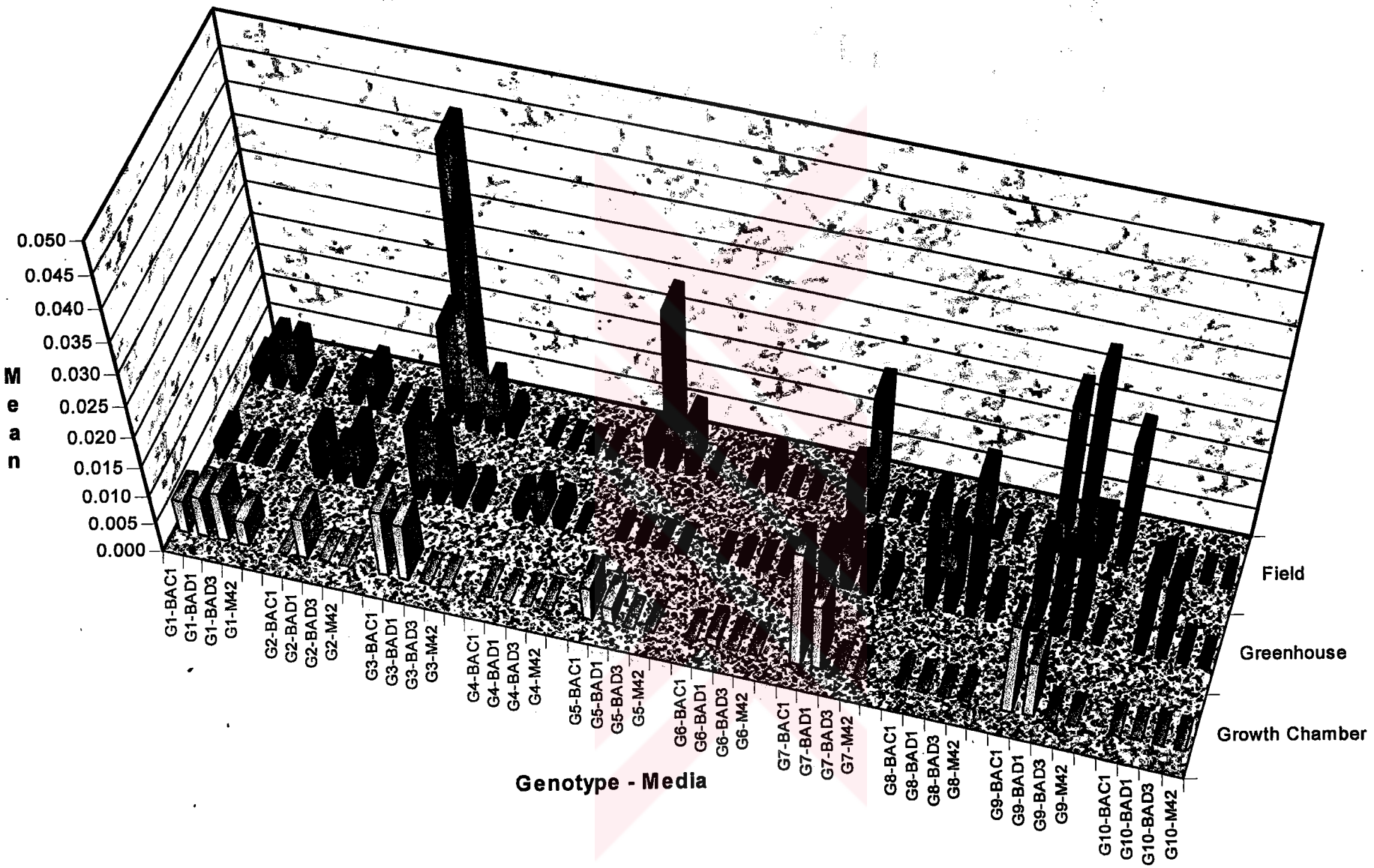
1) Effect of Genotype

The effect of genotype on callus production is given in Table 4.3. G-9 yielded the highest mean number of calli and was significantly better than all other genotypes except G-3. Again, G-6 had the lowest mean number of calli.

2) Effect of Growth Conditions

The effect of growth conditions on callus production is given in Table 4.4. Field-grown material gave the highest mean number of calli, but it was not significantly different from the greenhouse-grown material. However, the growth chamber-grown material yielded a significantly lower mean number of calli than the materials grown under the other two growth conditions.

Chart 4.4. The effect of genotype, growth condition, and media on anther response



3) Effect of Media

The effect of media on callus production is given in Table 4.5. Anthers cultured on BAD-1 medium produced the highest mean number of calli. However, BAC-1 was not significantly different from BAD-1. M-42 gave the lowest callus production.

4) The Effect of Genotype × Growth Condition Interactions

Genotype × growth condition interactions were highly significant for callus production (Table 4.3). Chart 4.5 shows that each genotype had a different mean for callus production when they were grown under different conditions.

5) The Effect of Genotype × Medium Interactions

Genotype × medium interactions were highly significant for callus production (Table 4.3). Chart 4.6 shows that the genotypes gave different means for callus production when cultured on different media.

6) The Effect of Growth Condition × Medium Interactions

Growth condition × medium interactions affected callus production significantly (Table 4.3). As shown in Chart 4.7, the effect of media differed when the plants were grown under different conditions.

7) The Effect of Genotype × Growth Condition × Medium Interactions

Genotype × growth condition × medium interactions affected the callus production significantly (Table 4.3). Chart 4.8 shows that the mean callus production by genotypes varied when they were grown under different conditions and cultured on different media. Each genotype had a different preference for media and growth conditions. The highest mean for callus production was obtained from field-grown G-9 when BAD-1 medium was used.

Chart 4.5. The effect of genotype and growth condition on callus production

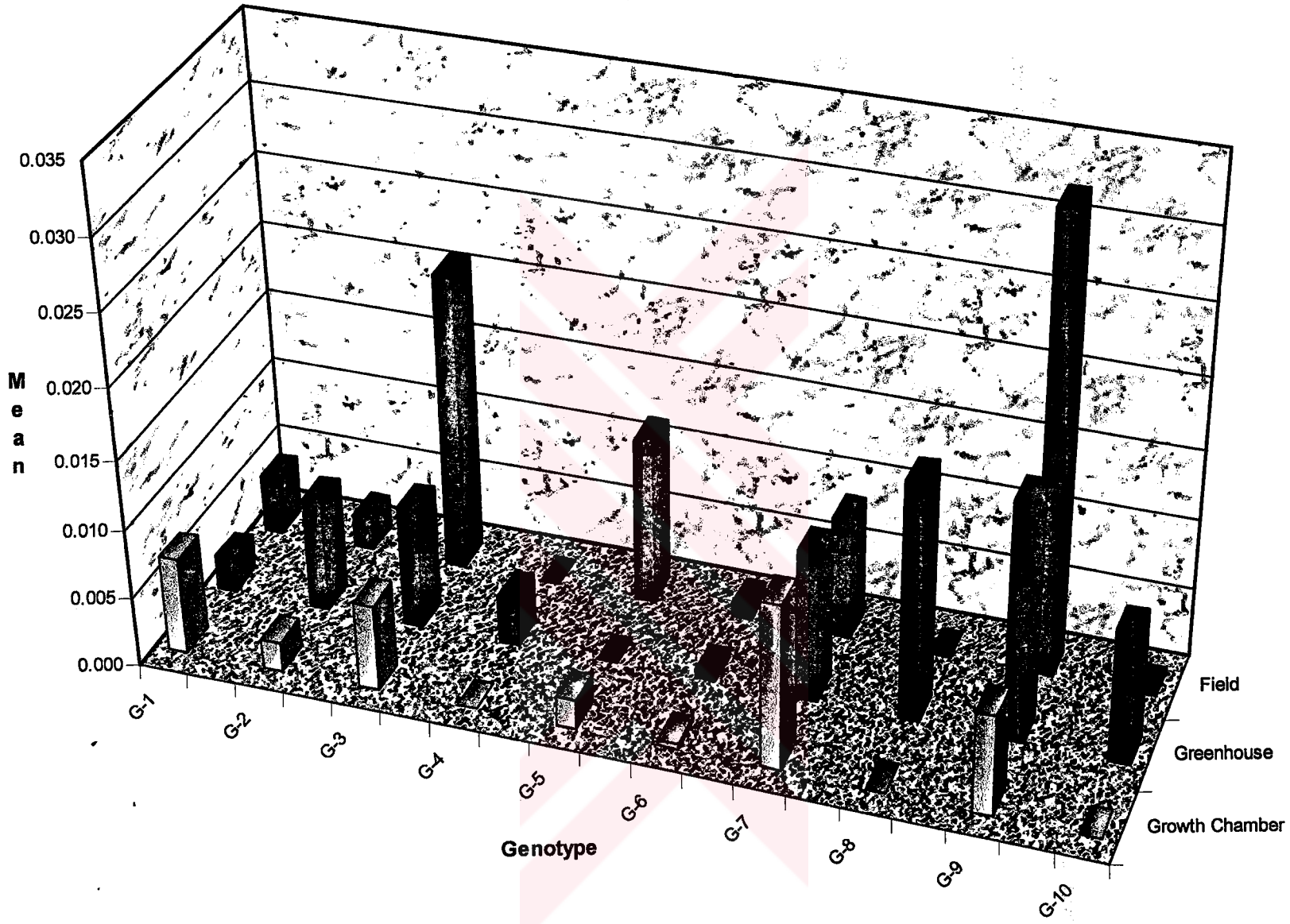


Chart 4.6. The effect of genotype and media on callus production

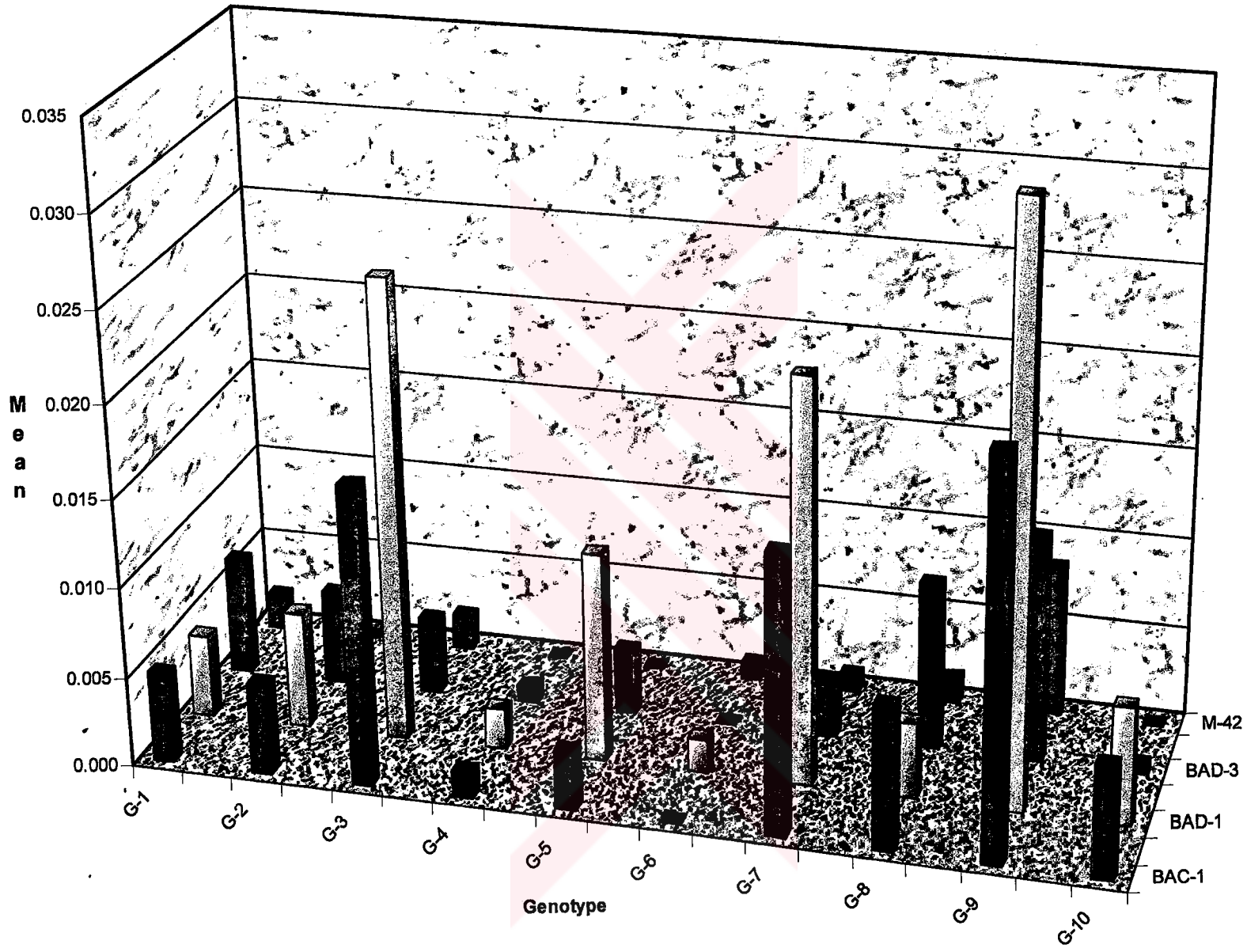


Chart 4.7. The effect of growth condition x media on callus production

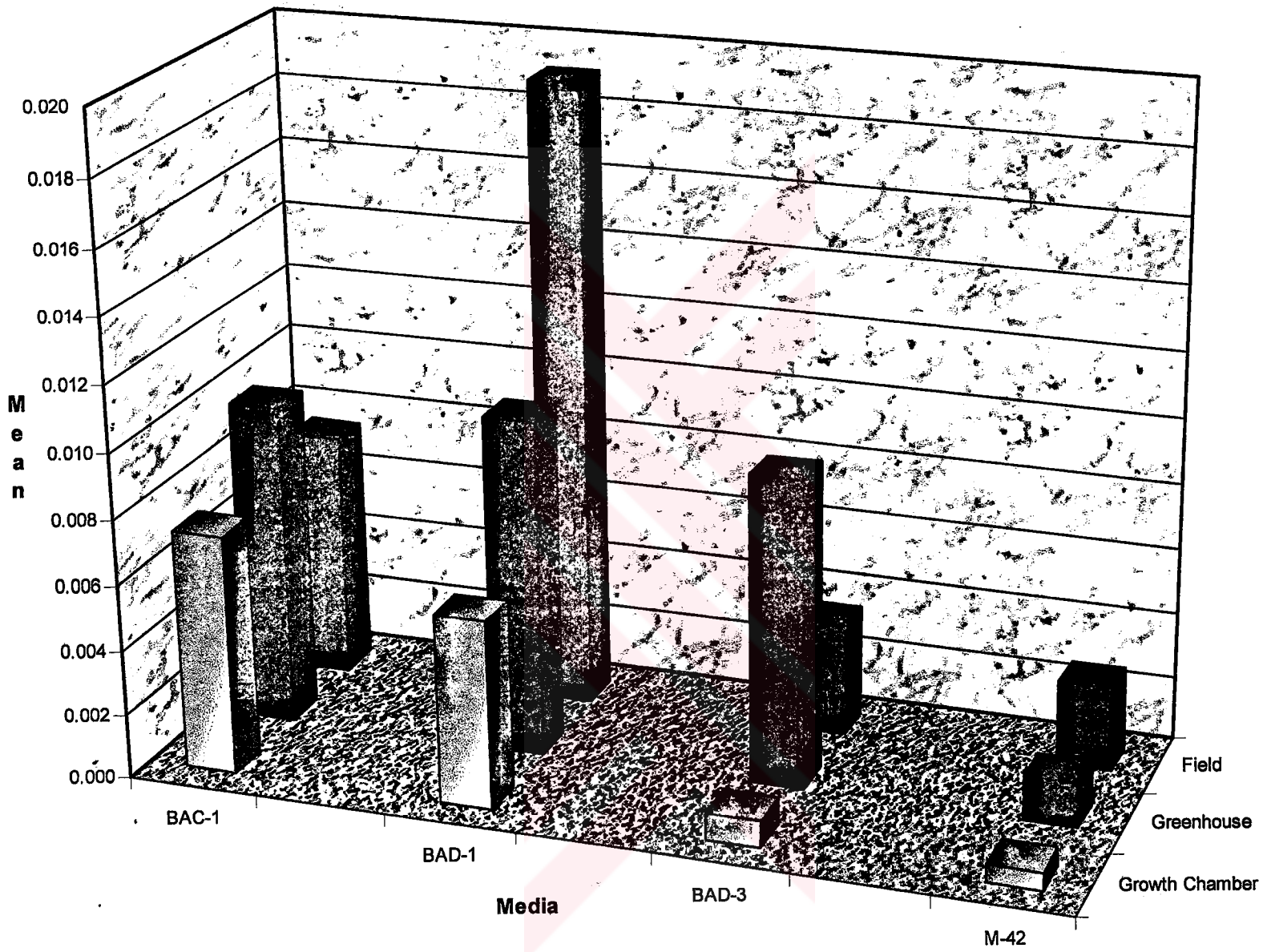
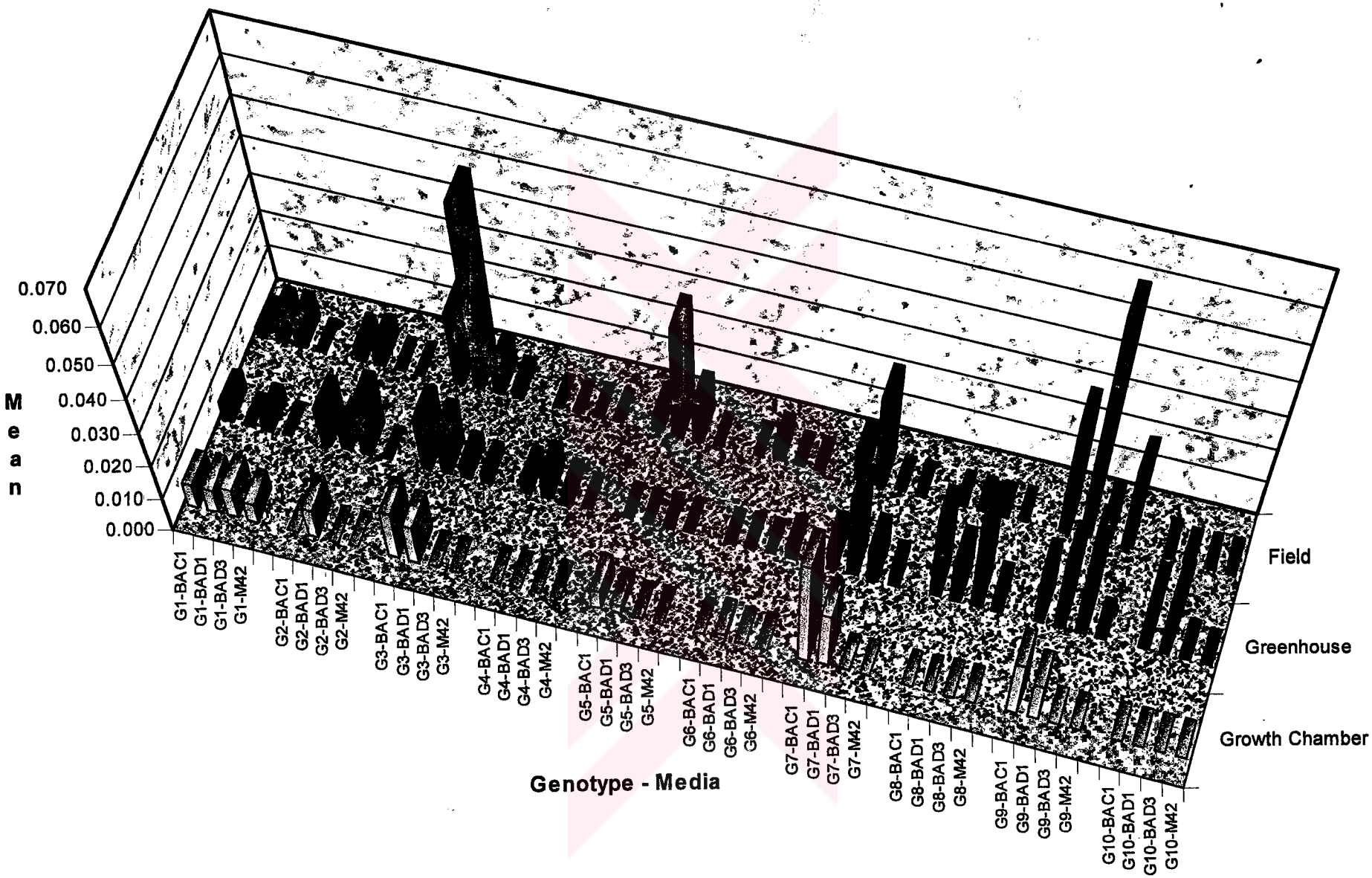


Chart 4.8. The effect of genotype, growth condition, and media on callus production



Cultures were then kept in the dark at 26°C until differentiation took place. Differentiation usually started within 7-10 days (Figure 4.5 B). Under dark conditions, some of the differentiated structures, i.e. shoot initials, started to develop chlorophyll (Figure 4.5 C). These cultures had been exposed to light only during the periodical observations. After shoot initiation, plantlets were transferred to modified MS media, and placed in a lighted growth room; some started developing chlorophyll within a day (Figure 4.5 D). Other plantlets turned green after additional exposure to light (Figure 4.5 E). After one to two weeks, calli showed an abundance of rooted plantlets (Figure 4.5 F), which were separated and subcultured. However, some plantlets never developed chlorophyll and remained albino (Figure 4.5 G). Seven to ten days later (Figure 4.5 H), the green plantlets were transferred to soil. In two cases, chimeric plants (green and albino plantlets together) originated from the same callus, probably from different tiller (axillary) primordia (Figure 4.5 I).

After transferring the plantlets to peat pellets, they were covered with plastic wrap for 3-4 days. The plastic wrap was opened a little each day to gradually harden and acclimatize the plants to normal conditions. When moved to greenhouse, the green plantlets showed variations in growth habits. Some of them tended to mature rapidly and developed spikes from first node (Figure 4.6 A). In addition, several morphological abnormalities were observed such as multiple spikes per tiller (Figure 4.6 B). These plants had various chromosome abnormalities, which will be discussed later in section 4.4. It is interesting to note that some regenerants, which developed from same callus, had different ploidy levels. Induced polyploids ($2n >$ 28 chromosomes) tended to be smaller than the parent tetraploid plant (Figure 4.6 C). Some pentaploid plant regenerants had spike morphology somewhat similar to that of hexaploid wheat (Figure 4.6 D). Neither polyploids nor aneuploids were fertile and did not set seed.

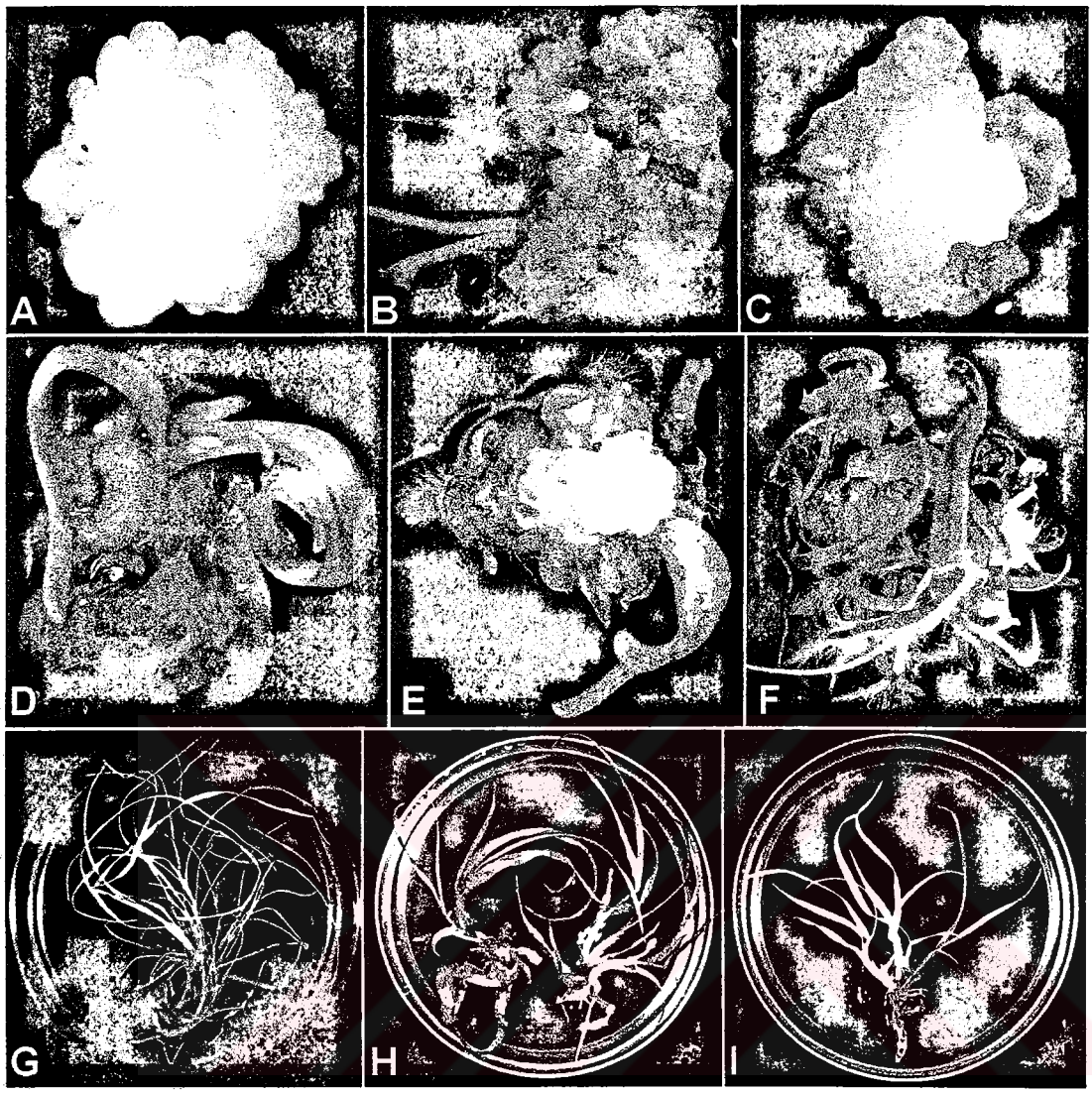


Figure 4.5. Callus differentiation and plantlet regeneration from anther culture of durum wheat. **A.** Callus transferred from induction medium to differentiation medium five weeks after culture. **B.** Ten days after transfer to differentiation medium, root and shoot initiation took place. **C.** Shoot initial developing chlorophyll under dark conditions. **D.** One day after transfer to lighted conditions; note the well-developed plantlet and the initiation of chlorophyll. **E.** One week after transfer to light; note dark-green leaflets. **F.** A mass of roots and shoots from a differentiated callus, six to seven weeks after initial culture. The plantlets were then separated and transferred to regeneration media. **G.** A well developed albino plantlet eight weeks after initial culturing of the anther; this plant did not develop any further. **H.** Healthy green plantlets on the regeneration medium, eight weeks after initial culturing of the anther. These plantlets are ready to be transferred to soil. **I.** Chimeric plants on regeneration medium originating from the same callus, probably from different primordial cells. This demonstrates the heterogeneity of the cells, induced by culture conditions and media, of the same callus.

Figure 4.6. Parental plants and anther culture-derived durum regenerants. **A.** Rapid development of an aneuploid ($2n$ = 36) plant. Note a spike (arrow) emerging close to the crown; also note the relative size of the plant (the side of the pot is 13 cm). **B.** Another aneuploid plant ($2n$ = 38) that developed a spike at the node (arrow) and had few leaves. **C.** Normal tetraploid ($2n$ = 28) durum wheat plant (left) and a pentaploid ($2n$ = 35) plant (right), both of the same cultivar (G-9). **D.** Another pentaploid ($2n$ = 35) plant, with an extra B-genome, showing hexaploid wheat-like spikes (arrow).

Overall, 324 plants were regenerated from the 86,400 anthers cultured (0.38%). 76 albino plants (0.09%) and 248 green (0.29%) were obtained (Table 4.1).

4.3.2.1. Green Plant Regeneration

Of the 324 plants regenerated, 248 developed chlorophyll and turned green. Thus, 76.5% of the total plants were green (Table 4.1). ANOVA results for green plant regeneration of the ten durum wheat genotypes, four media, and three growth conditions are given in Table 4.2. As shown in Table 4.3, genotypic effect for green plant regeneration was highly significant, the effect of growth condition was significant, and genotype × growth condition interactions were also highly significant.

1) Effect of Genotype

Only three of the ten genotypes produced green plants. The effect of genotype on green plant regeneration is given in Table 4.3. G-9 gave the highest mean number of green plants. However, it was not significantly different from G-7, but significantly different from G-3. G-7 and others were not significantly different from each other.

2) Effect of Growth Condition

Green plants were obtained only from field-grown and greenhouse-grown donor material. The effect of growth conditions on green plant regeneration is given in Table 4.4. Field-grown materials apparently yielded higher green plant regenerants than the greenhouse-grown materials, and differences were significant. No green plants were obtained from growth chamber-grown material.

3) Effect of Media

Green plants were obtained from anthers cultured on M-42, BAD-1, and BAD-3 media (Table 4.5). The modified M-42 gave the highest number of green plant regenerants, followed by BAD-1 and BAD-3. No green plants were obtained

from calli and/or embryoids that were initiated on BAC-1 medium. However, statistical analyses revealed no significant difference among the media used for green plant regeneration (Table 4.2).

4) Genotype × Growth Condition Interactions

Genotype × growth condition interactions affected the mean for green plant regeneration (Table 4.2). Chart 4.9 shows that the three genotypes that generated green plants had a different mean yield when the donor plants were grown under different conditions. All three genotypes gave a higher mean for green plants when the donor plants were grown under field conditions.

4.3.2.2. Albino Plant Regeneration

Of the 324 plants regenerated, 76 plants did not develop chlorophyll and remained albino, i.e., 23.5% of total plants regenerated (Table 4.1). ANOVA results for albino plant regeneration of the ten durum wheat genotypes, four media, and three growth conditions are given in Table 4.2. Only growth conditions had significant effect on albino plant regeneration.

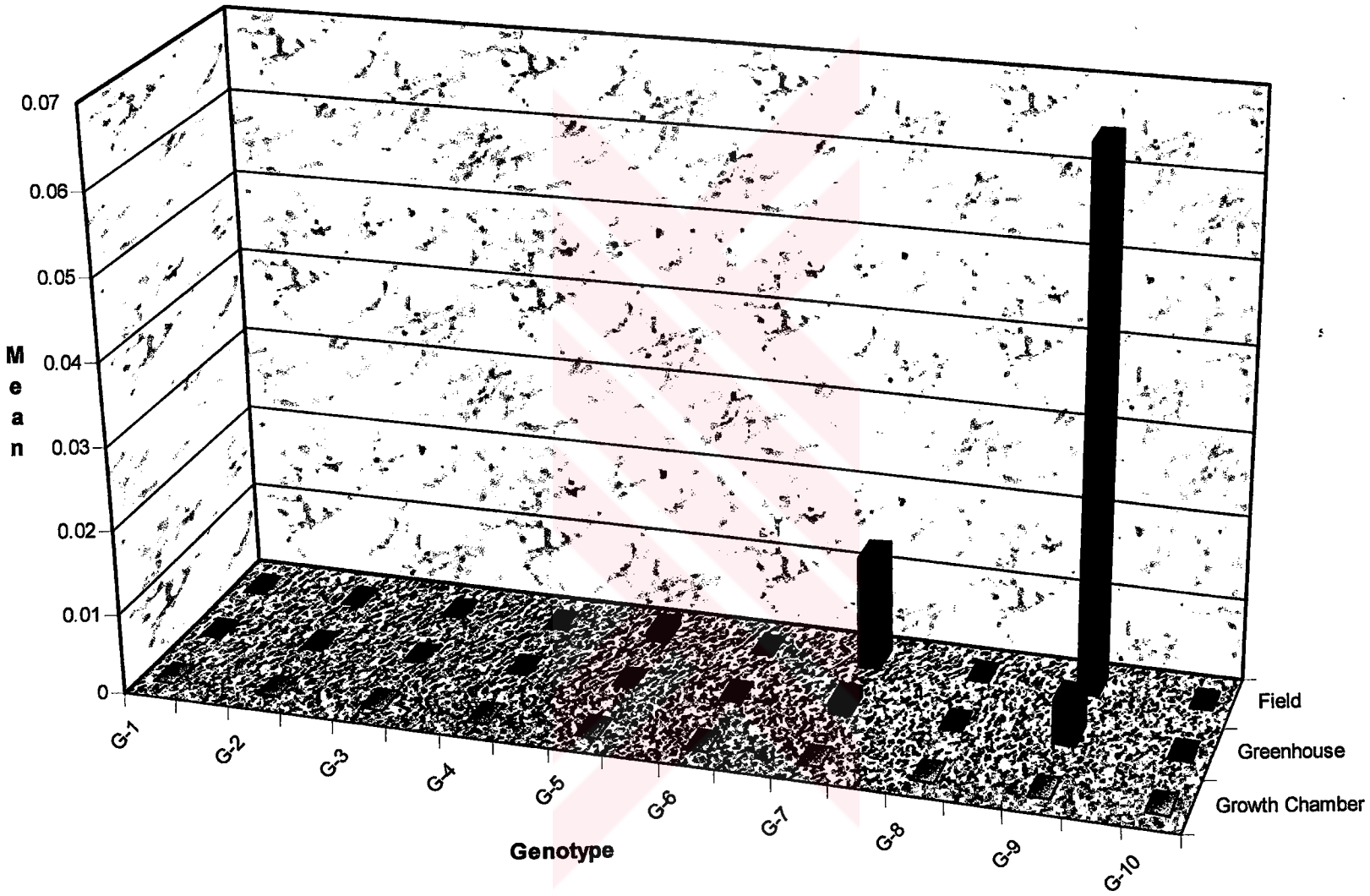
1) Effect of Genotype

Albino plants were obtained from seven of the ten genotypes. The effect of genotypes on albino plant regeneration is given in Table 4.3. G-3 gave the highest mean number of albino plants, but differences among genotypes were not significant.

2) Effect of Growth Condition

Albino plants were obtained from both greenhouse- and field-grown materials. No albino plants were obtained from growth chamber-grown material. The effect of growth conditions on albino plant regeneration is given in Table 4.4. Greenhouse-grown material gave the highest mean number of albino plants, but it was not significantly different from field-grown material.

Chart 4.9. The effect of genotype and growth condition on green plant regeneration



3) Effect of Media

Albino plants were obtained from anthers cultured on all four media. The effect of media on albino plant regeneration is given in Table 4.5. BAD-3 gave the highest mean number of albino plants, and BAC-1 the lowest. However, there was no significant difference in albino plant regeneration on the four media (Table 4.2).

4.4. Cytological Study of Regenerants

A total of 133 plants were studied for chromosome number (Table 4.6) and structural changes. Haploids, disomics (tetraploids), polyploids, and aneuploids were recorded. Structural abnormalities such as translocations, telocentrics, acentric fragments, Robertsonian translocations, dicentrics, and chromatid exchanges were observed.

Table 4.6. Chromosome numbers of anther culture-derived plants at mitotic or meiotic metaphase.

	Chromosome number (2*n*) of a cell									
	14	**28**	**35**	**36**	**37**	**38**	**39**	**42**	**63**	**70**
No. of albino plants	7	24	0	0	0	0	0	0	0	0
No. of green plants	0	52	22	9	4	5	2	6	1	1

4.4.1. Conventional Techniques

Of the 324 regenerants, 133 were analyzed for chromosome numbers and chromosomal aberrations. A total of 20-25 cells were scored from each plant.

4.4.1.1. Albino Plants

31 of the 76 albino plantlets were scored for chromosome number using conventional techniques. Of these, 7 plantlets were haploid (Figure 4.7 A) and 24 were tetraploid (Figures 4.7 B, C, and D) (Table 4.6). Several chromosomal abnormalities were observed in the tetraploid albino plants. These included telocentric chromosomes, dicentric chromosomes (Figure 4.7 C and D), and other abnormalities like lightly stained regions (Figures 4.7 B and C).

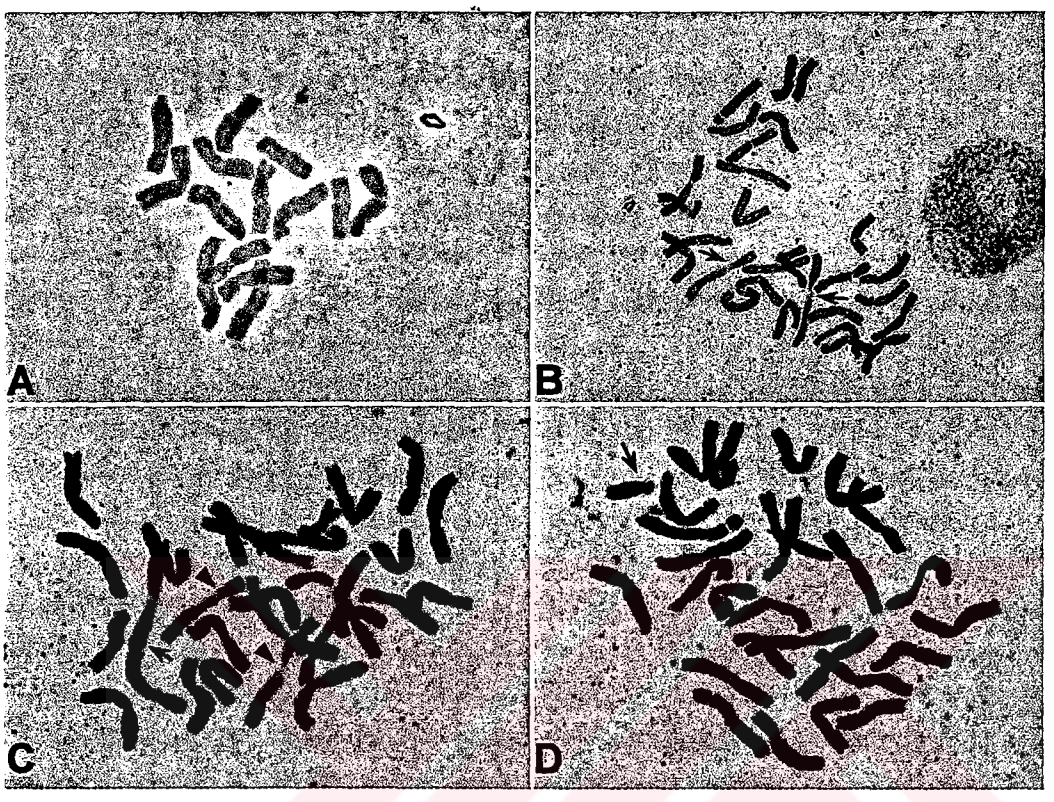


Figure 4.7. Somatic metaphase chromosomes from root-tip cells of anther culture-derived albino durum plantlets. **A.** A haploid ($2n$ = 14) with no apparent chromosome abnormalities. **B.** 28 chromosomes of a spontaneously doubled plant; two chromosomes exhibit lightly stained regions (LSRs) (arrows). **C.** 28 chromosomes of a spontaneously doubled plantlet. Note the dicentric chromosome (arrow) involving one of the satellited chromosomes, and two chromosomes exhibiting LSRs (arrowheads). **D.** 28 chromosomes of another spontaneously doubled plant; a telocentric chromosome (arrow) and four satellited chromosomes (1B and 6B) are clearly visible.

six hexaploid ($2n = 6x = 42$), one nonaploid ($2n = 9x = 63$), and one decaploid ($2n = 10x = 70$). Aneuploid plants with $2n = 36$ to 39 chromosomes were also obtained (Figures 4.8 B-F) with various structural abnormalities, i.e. telocentrics, fragments, and sticky ends. Meiotic cells of anther culture-derived polyploids (Figure 4.9 A) and aneuploids (Figure 4.9 B) showed various pairing configurations such as trivalents, quadrivalents, pentavalents, and hexavalents, which were investigated using fluorescent GISH.

4.4.2. Fluorescent GISH Studies

Fluorescent GISH proved to be an excellent technique for identifying the A-genome and B-genome chromosomes in both mitotic and meiotic cells. In mitotic metaphase cells, GISH elucidated intergenomic translocations, chromatid exchanges, and preferential multiplication of chromosomes of one genome. By using morphological landmarks, precise chromosome duplications were revealed. For example, the characteristic 4A·5A·7B translocation (Naranjo, 1990) was detectable, although the translocated portion from 5A was not seen using this technique. In meiotic metaphase I cells, GISH helped to identify inter- and intra-genomic chromosome pairing configurations. However, rearrangements involving chromosomes of the same genome could not be detected, which is a typical disadvantage of GISH.

4.4.2.1. Fluorescent GISH of Mitotic Cells

1) Preferential Multiplication of B-genome Chromosomes

Green plants with 28 chromosomes were normal in genomic constitution, i.e., with 14 A-genome and 14 B-genome chromosomes (Figures 4.10 A and B). However, in most polyploid cells, there was a preferential multiplication of B-genome chromosomes (Table 4.7). Figures 4.10 C and D, and,

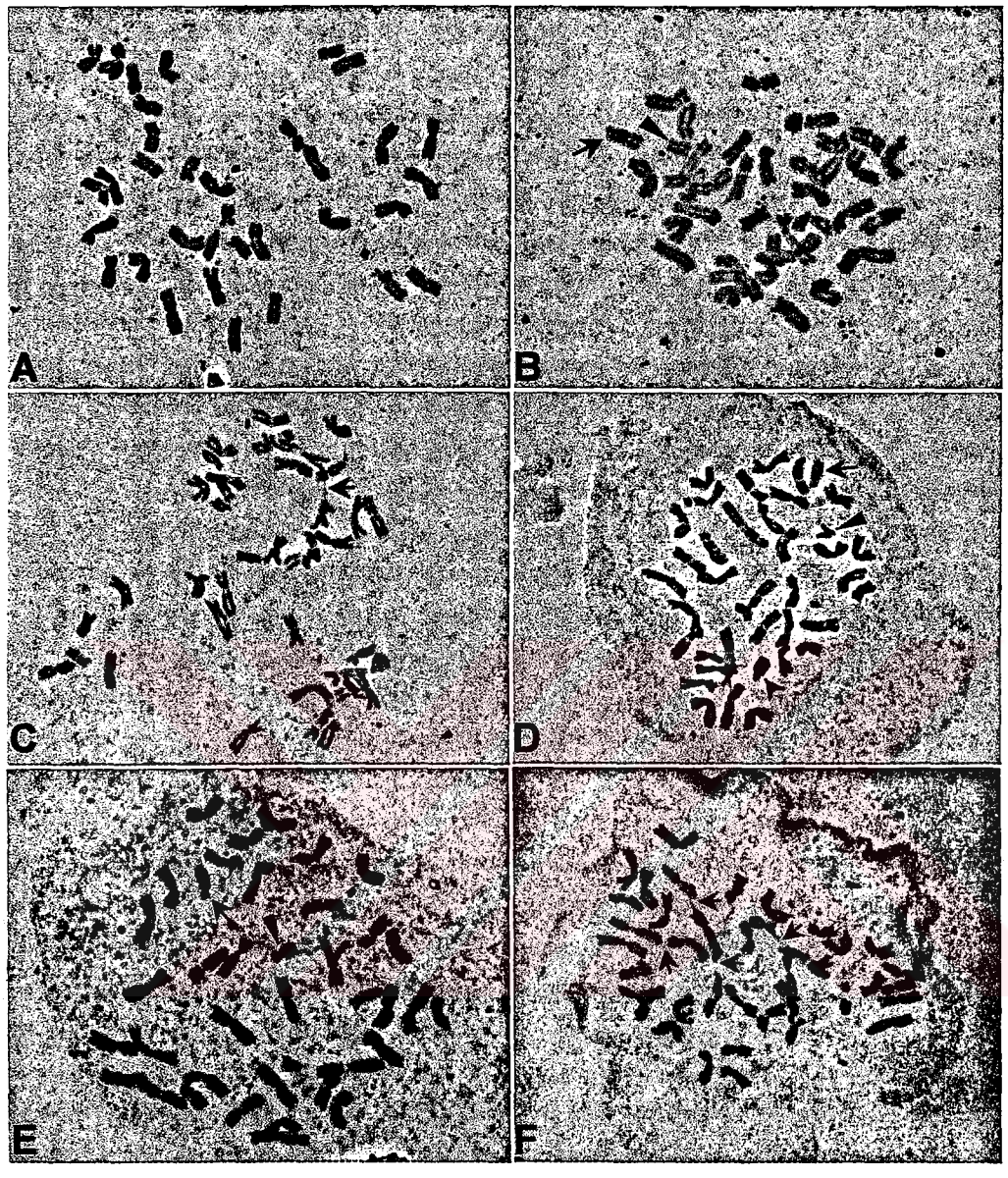


Figure 4.8. Somatic metaphase chromosomes from root-tip cells of anther culture-derived green plantlets. **A.** A pentaploid ($2n$ = 35) cell. **B.** Aneuploid (2n = 38) showing the loss of chromatin from one chromatid (arrow), and a diminished acrocentric chromosome (arrowhead). **C.** Another aneuploid ($2n$ = 39) showing fused chromatids (arrow). **D.** An aneuploid ($2n$ = 39) with multiple aberrations; note an acrocentic (arrow), a fragment (arrowhead), and a telocentric (V-shaped arrowhead). **E.** A 39-chromosome cell with a clear acrocentric chromosome (arrow) and a telocentric chromosome (arrowhead). **F.** An aneuploid cell showing fusion (stickiness) of chromatids of several chromosomes (arrows).

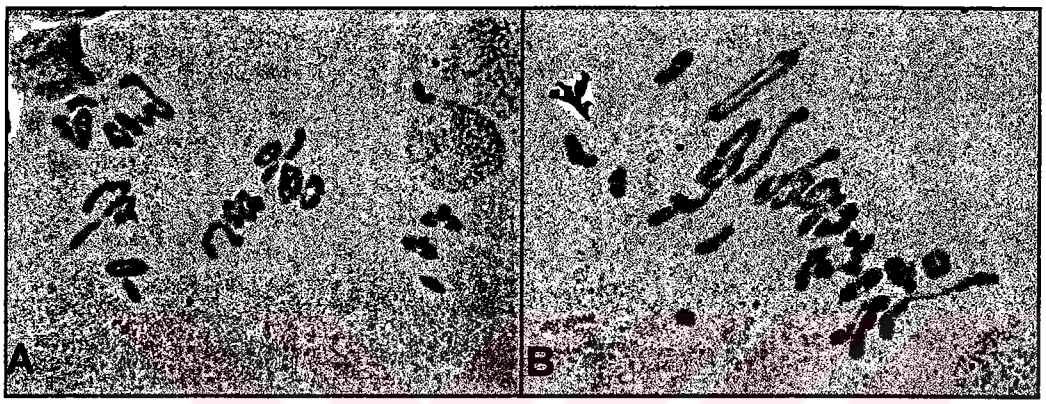


Figure 4.9. Meiosis of anther culture-derived green polyploid and aneuploid durum plants. **A.** A 42-chromosome cell showing 1 IV + 1 frying pan III + 1 heteromorphic III + 13 ring II + 2 rod II + 2 I. This cell was later analyzed using fluorescence to determine genomic constitution (see Figures 13 E and F). **B.** A 39-chromosome cell showing 2 III + 10 ring II + 4 rod II + 5 I.

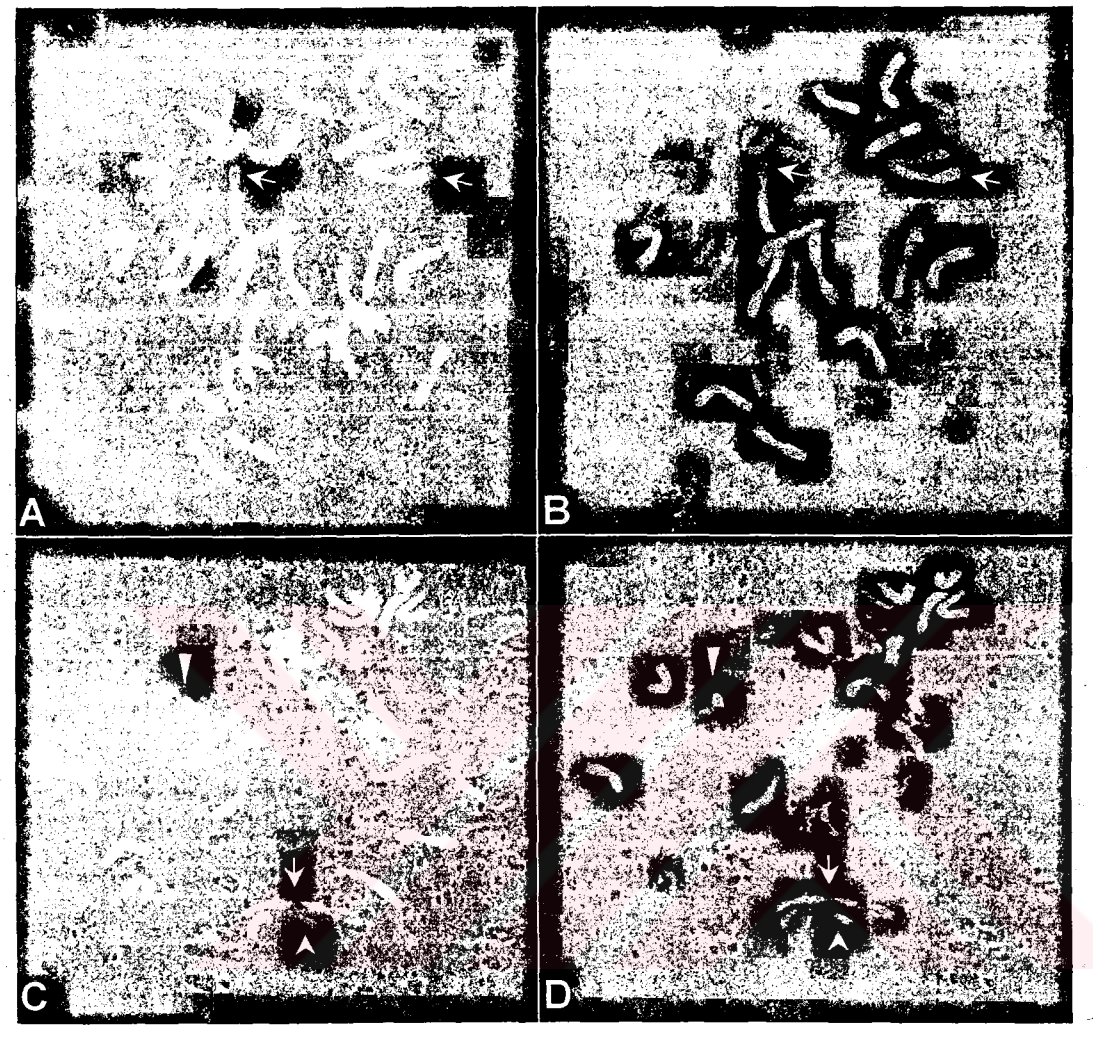


Figure 4.10. Fluorescent genomic *in situ* hybridization of somatic chromosomes of anther culture-derived green durum plants. **A.** 28-chromosome cell counterstained with propidium iodide (PI). **B.** Same cell as in A hybridized with total genomic DNA of *T. urartu* and detected with fluorescein isothiocyanate (FITC). Chromosome 4A is detectable by the evolutionary translocation from 7B (arrows). **C.** A pentaploid ($2n = 35$) cell counterstained with PI. **D.** Same cell as in C, showing 12 A-genome chromosomes + an A-genome fragment (V-shaped arrow) detected with FITC; note the fused chromosomes (arrow) and an intergenomic translocation (arrowhead).

Figures 4.11 A and B for example, show pentaploid cells, each with 14 chromosomes of the A-genome and 21 of the B-genome.

2) Structural Chromosomal Abnormalities

In addition to numerical abnormalities, various structural abnormalities were also detected. Robertsonian translocations (Figures 4.11 B and D), acrocentrics (Figure 4.11 C), terminal translocations (Figure 4.11 E), and chromatid exchanges (Figure 4.11 F) were observed. Interstitial translocations were also observed, albeit rarely. Intergenomic chromatid exchanges, both terminal and interstitial, were observed. Fragments of A-genome chromosomes were more frequent than those of the B-genome.

Table 4.7. The A- and B-genome chromosome number of individual cells at mitotic and meiotic metaphase elucidated by fluorescent GISH.

	Chromosome number ($2n$) of a cell									
	14	**28**	**35**	**36**	**37**	**38**	**39**	**42**	**63**	**70**
No. of A-genome chromosome	-	14	14	14	14	14	14	14	-	-
No. of B-genome chromosome	-	14	21	22	23	24	25	28	-	-

4.4.2.2. Fluorescent GISH of Meiotic Cells

1) Intergenomic and Intragenomic Chromosome Configurations

Some tetraploid green plants looked normal at meiotic metaphase I with 7 A-genome bivalents and 7 B-genome bivalents (Figures 4.12 A and B). However, univalents

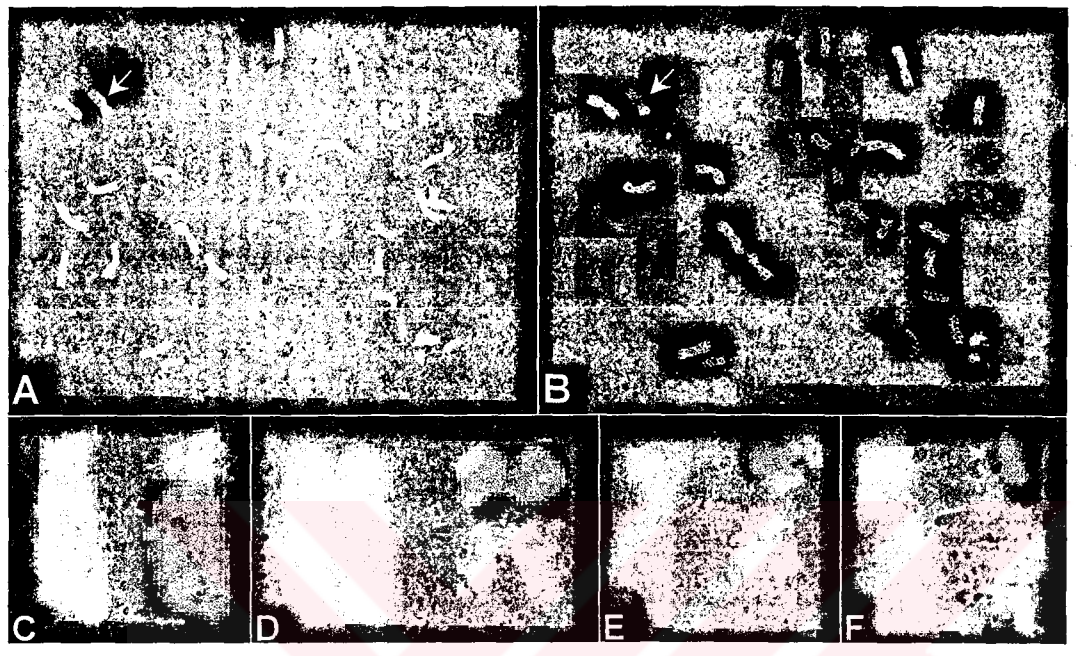


Figure 4.11. Fluorescent genomic *in situ* hybridization of somatic chromosomes of anther culture-derived green durum plants and some chromosome configurations selected from different cells to show the range of configurations and aberrations. **A.** Pentaploid ($2n = 35$) cell counterstained with propidium iodide (PI). **B.** Same cell as in A hybridized with total genomic DNA of *T. urartu* and detected with fluorescein isothiocyanate (FITC). A Robertsonian translocation is clearly visible (arrow). Also note 14 A-genome chromosomes and 21 B-genome chromosomes. **C.** An acrocentric hybrid A-genome chromosome counterstained with PI (left), the short arm coming from B-genome (right) detected with FITC. **D.** A PI counterstained chromosome (left) showing a Robertsonian translocation detected with FITC (right). **E.** Close-up of the Robertsonian translocation from B. **F.** A 4',6-diamidino-2-phenylindole (DAPI) counterstained chromosome (left) showing chromatid exchange involving A-genome and B-genome chromatids detected with FITC (right).

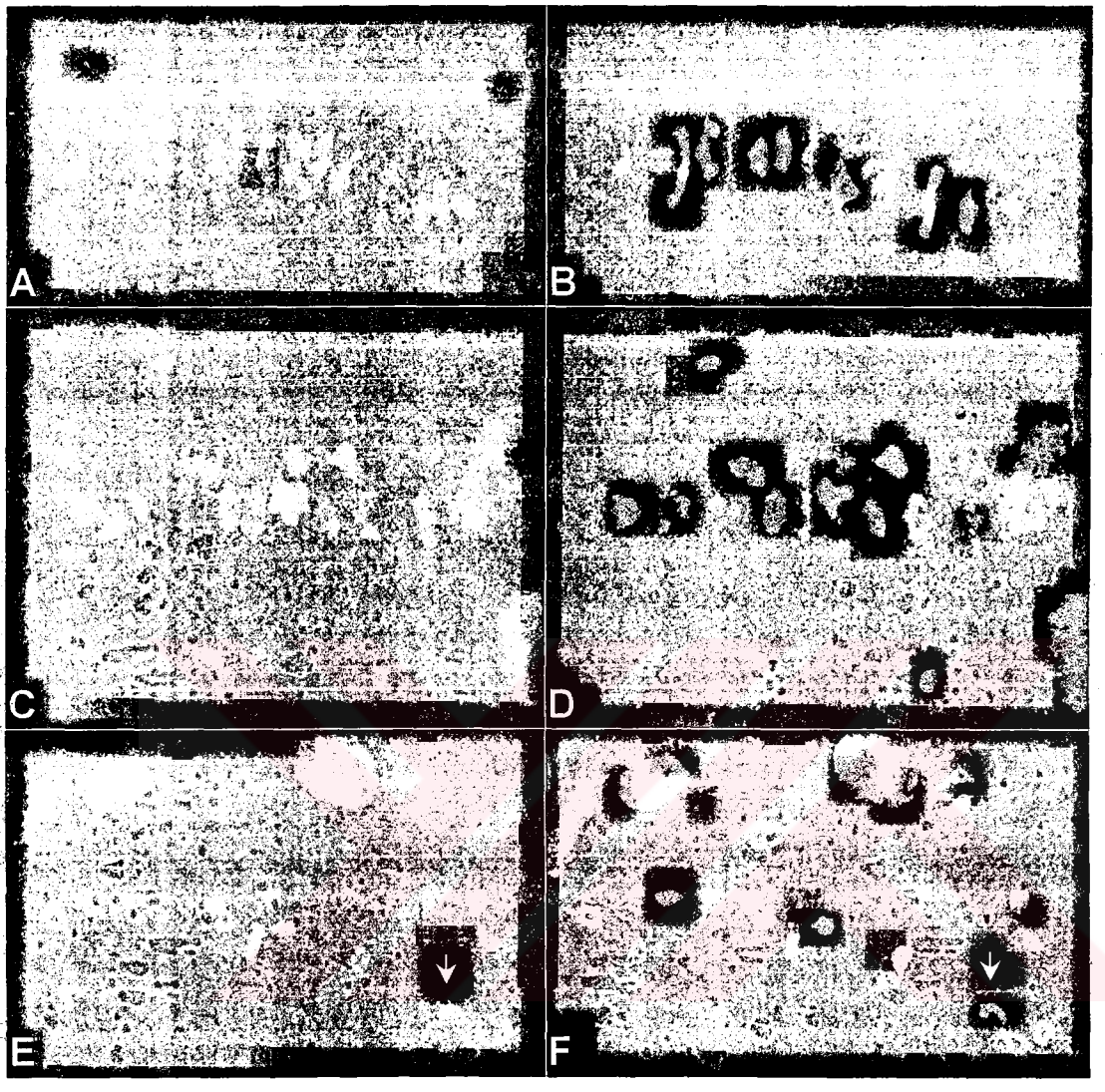


Figure 4.12. Fluorescent genomic *in situ* hybridization of meiotic chromosomes of anther culture-derived green plants. **A.** Propidium iodide (PI) counterstained cell with 14 bivalents (11 ring II + 3 rod II). **B.** Same cell as in A, where the A-genome chromosomes were detected using fluorescein isothiocyanate (FITC). Note seven bivalents from each genome. **C.** 12 bivalents and 4 univalents of a green plant counterstained with PI. **D.** Same cell as in C showing 6 bivalents and 2 univalents from each genome detected with FITC. **E.** Another cell with 12 ring bivalents and 1 ring quadrivalent (arrow) counterstained with PI. **F.** Same cell as in E showing 6 A-genome ring bivalents, 6 B-genome ring bivalents, and an intergenomic ring quadrivalent (arrow) detected by using FITC.

(Figures 4.12 C and D) and intergenomic quadrivalents (Figures 4.12 E and F) also occurred in several tetraploid plants. Preferential multiplication of B-genome chromosomes was observed in meiotic cells also (Figures 4.13 A-F). Intragenomic and intergenomic rod and ring bivalents, some heteromorphic (Figure 4.14 A), were observed. Many of the V-shaped trivalents were of the same genome (Figures 4.14 C, D and E); some of these appeared to be heteromorphic (Figures 4.14 D and E).

2) Structural Abnormalities

Clear terminal (Figure 4.14 B) and Robertsonian translocations between the A and B genome chromosomes were recorded, but interstitial translocations were never observed. Frying-pan trivalents (Figures 4.14 F and G) involved both the A- and B-genome chromosomes. Some of these frying-pans were due to translocations (Figure 4.14 G). Quadrivalents were almost always comprised of chromosomes of both genomes (Figures 4.14 H, I and J), and many were clearly due to translocations. Intergenomic ring quadrivalents (Figure 4.14 K), and higher multivalents, e.g., pentavalents (Figures 4.14 L and M) and hexavalents were also observed. Chromatid breaks as well as non-sister chromatid exchanges were recorded. Fluorescent GISH has the ability to reveal only non-sister chromatid exchanges involving both A- and B- genome chromosomes.

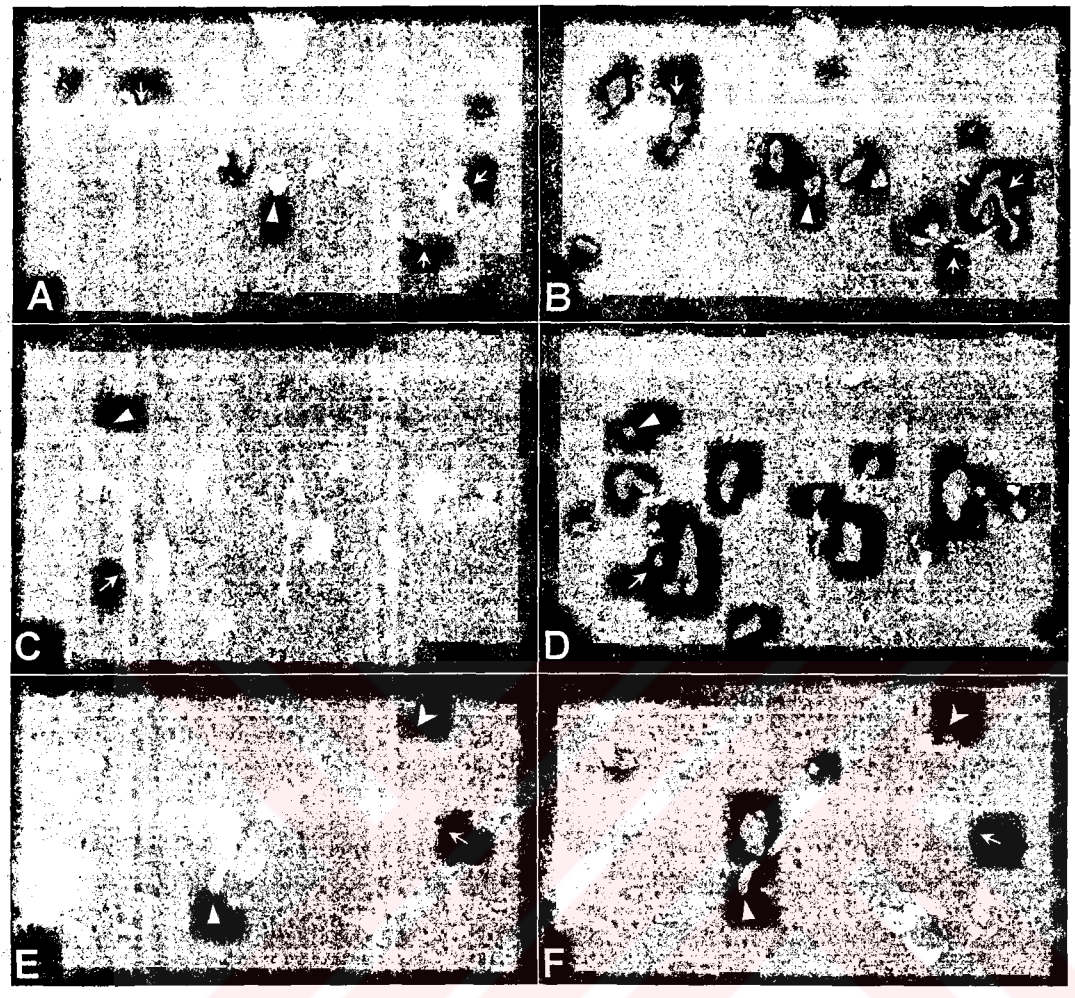


Figure 4.13. Fluorescent genomic *in situ* hybridization of meiotic chromosomes. **A.** Propidium iodide (PI) counterstained aneuploid ($2n = 37$) cell. **B.** Same cell as A after hybridization with *T. urartu* genomic DNA, detected with fluorescein isothiocyanate (FITC). Note the A- and B-genome quadrivalents (arrows) and intergenomic ring bivalent (arrowhead). **C.** PI-counterstained cell showing a pentavalent. **D.** Same cell as in C hybridized with *T. urartu* genomic DNA and detected with FITC. The pentavalent is a clear result of a translocation of A-chromatin on to a B-genome chromosome (arrow). Also note the A-genome fragment (arrowhead) **E.** A cell counterstained with PI (1 IV + 1 frying-pan III + 1 heteromorphic V-shaped III + 13 ring II + 2 rod II + 2 I). **F.** The same cell as in E after hybridization, showing three translocations; one involving the formation of a frying pan III (V-shaped arrowhead), a Robertsonian translocation as part of an A-genome quadrivalent, and a rod II with a telomeric translocation. Note the heteromorphic A-genome rod (arrowhead) and a heteromorphic B-genome trivalent (arrow). This cell has 2 doses of the A-genome and 3 doses of the B-genome .

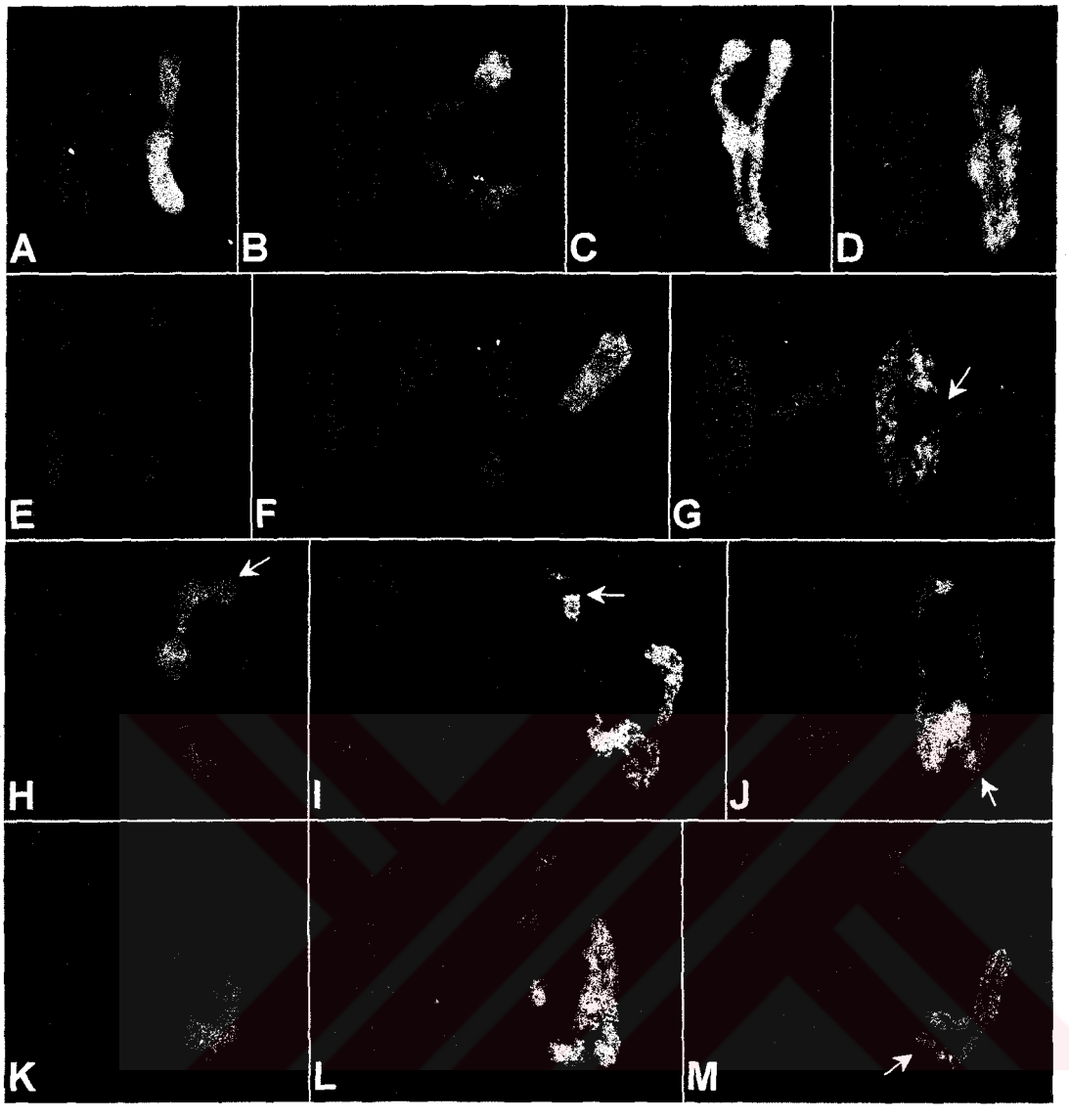


Figure 4.14. Fluorescent genomic *in situ* hybridization of chromosome configurations selected from different cells to show the range of formations and aberrations. Each figure is represented by a propidium iodide-counterstained chromosome or configuration (shown on left), along with the same chromosome (right) probed for the A-genome using *T. urartu* genomic DNA labeled with fluorescein isothiocyanate, which fluoresces as bright green. The faded chromosomes or chromosome segments are of the B-genome. **A.** Heteromorphic A-genome rod bivalent due to the loss of chromatin of one chromosome (appears as an acrocentric chromosome in somatic spreads). **B.** B-genome rod bivalent showing a clear telomeric translocation from the A-genome. **C.** A-genome trivalent from a cell with more than 14 A-genome chromosomes, a good indication that all 3 are homologous. **D.** Heteromorphic A-genome trivalent. **E.** Heteromorphic B-genome trivalent. **F.** BBA-genome frying-pan trivalent. demonstrating intergenomic pairing. **G.** A clear B-genome translocation (arrow), resulting in an AAB-genome frying-pan trivalent. **H.** AABB-genome quadrivalent clearly due to an A-genome fragment translocated (arrow) onto a B-genome chromosome. The translocation chromosome is probably the same as in the rod bivalent in Figure above B. **I.** A-genome quadrivalent involving a chromosome that resulted from a Robertsonian translocation (arrow). **J.** Another quadrivalent resulting from a translocation of A-genome chromatin to a B-genome chromosome (arrow). **K.** A ring quadrivalent apparently resulting from a very small telomeric exchange from the B-genome to the A-genome. **L.** AAABB-genome pentavalent with the same translocation chromosome as in Figure B and H. **M.** Inter- and intragenomic pentavalent with 3 A-genome chromosomes and 2 B-genome chromosomes, a result of a translocation (arrow) as also shown in the other figures.

5. DISCUSSION

Anther culture is being increasingly used in wheat breeding programs both as a source of haploids (Kisana et al., 1993; Baenziger, 1996; Stober and Hess, 1997) and new genetic variation (Hu, 1986, 1997; Lu et al., 2000). Doubled haploids provide a shortcut to breeding and have interested plant breeders for the past several decades. The breeding method involves making haploid tissues or haploid plants from heterozygous parents, and doubling the chromosomes in the regenerant tissues or plants to obtain homozygous lines (Baenziger, 1996; Khush and Virmani, 1996). There are numerous factors that trigger androgenesis, and with the recent improvement of various culture techniques, it has become possible to induce pollen calli/embryos in a large number of plant species. These factors can be genetic, physiological, or chemical. Any one of these factors, or a combination of them, can induce the pollen grains to enter into a new development pathway. Embryogenesis from isolated microspores has a number of important applications to crop improvement, including *in vitro* mutant selection, transformation, and production of homozygous lines in practical breeding. Production of haploid cultures may prove useful in biotechnological manipulations, including RFLP analyses and specific gene transformations (Sopory and Munshi, 1996). The anther culture system has been a favorable choice of many researchers because of the availability of a large number of microspores within each anther that could potentially produce haploid plants. For many plant species, anther culture is the most efficient means of obtaining haploid plants for use in plant breeding (Zhou, 1996).

The anther-culture ability of tetraploid wheats has not been studied systematically. Defining the factors affecting anther culture could increase the use of *in vitro* techniques in durum wheat breeding. In earlier studies, the response of different tetraploid wheat cultivars to androgenesis was very low and few embryoids were obtained in some cultivated varieties (Foroughi-Wehr and Zeller, 1990). In one study, only a few albino plants (Zhu et al., 1979) were obtained, two green plantlets were recovered in another study (Hadwiger and Heberle-Bors, 1986), and five green plantlets from yet another study (Ghaemi et al., 1993b). In the present extensive

study, durum anther-culture ability was influenced by genotype, growth condition, and media formulations. These factors are discussed in detail.

5.1. Genotypic Control of Anther-Culture Ability

Although all ten genotypes we studied showed anther-culture response and produced calli and/or embryoids, significant genotypic differences in yield were observed; G-9 was the best-responding cultivar with 1.28 responding anthers per 100 anthers cultured. Anther culture is known to be strongly influenced by the genotype of the donor plant (Lazar et al., 1987, 1990; Agache et al., 1989; Pauk et al., 1991; Ghaemi et al., 1993a, b; Moieni and Sarrafi, 1995; Moieni et al., 1997a). Szakács et al. (1988) found that the induction of callus and regeneration of green plants were controlled by multigenes. Ekiz and Konzak (1994a) reported that callus induction, plant regeneration, and green plant development were controlled by different genetic mechanisms. These workers reported both nuclear and cytoplasmic control of anther-culture response in wheat, although the majority of the variation was probably caused by nuclear genes (Ekiz and Konzak, 1991a, b, c, 1994b). Earlier, Sagi and Barnabas (1989) also reported cytoplasmic and nuclear effects on *in vitro* embryogenesis of anther culture of hexaploid wheat, but no cytoplasmic-nuclear interactions were found.

5.2. Albino and Green Regenerants

5.2.1. Albino Haploids

After culturing 86,400 anthers in 10 durum cultivars in the present study, not a single green haploid plantlet was obtained; only albino haploids were produced. Earlier studies have shown that the frequencies of green plantlets regenerated from anther culture vary within and between plant species. Green plant regeneration from anther culture of cereal crops is both a heritable and environmentally influenced characteristic (Zhou, 1996). The low yield of haploid green plants from wheat anther culture is a serious problem and a limiting factor in the use of anther culture (Andersen et al., 1987; Zhou and Konzak, 1992; Otani and Shimada, 1995). Several

studies have shown that the production of green plants from anther culture of cereal crops is under the control of complex genetic factors (involving both additive and non-additive gene action) as well as some cytoplasmic factors (Sagi and Barnabas, 1989; Tuvesson et al., 1989; Ekiz and Konzak, 1991a, b, c).

The primary cause of albinism is not yet understood. Many factors have been found to affect the degree of albinism, such as the genotype and physiological state of the donor plants, the developmental stage of the microspores, culture temperature and cold pretreatment. Albino plants of barley, for example, do not contain mature chloroplasts and in rice there is a large-scale deletion of the plastid genome in microspore-derived albino plants (see Jahne and Lörz, 1995). It may be assumed that both nuclear and cytoplasmic genes are needed for the production of green haploid plantlets in durum wheat. The lack of or deficiency in the plastid genome of microspores may well contribute to the development of albino haploid plantlets in durum wheat. Although the cultured microspores will presumably have intact nuclear genes, they may not always have the cytoplasmic factors to interact with and would therefore be albino. This hypothesis is supported by the fact that durum wheat × maize hybridizations produce only green haploids (Almouslem et al., 1998), presumably because they contain the full plasmon of the female durum parent. It may be possible to verify this hypothesis by culturing ovules to produce haploids. If cytoplasmic factors are indeed essential for green haploid production, then gynogenetic haploids would be green.

5.2.2. Green Regenerants

Although no green haploids were recovered in the present study, green regenerants at different ploidy levels ranging from $2n = 28$ to 70 chromosomes were obtained. We generated the highest mean number of anther culture-derived green plants thus far reported. Yet the green plant regeneration in durum wheat (0.29%) was much lower than that obtained in bread wheat (3.2%, Kasha et al., 1990; 2-4.5%, Orshinsky et al., 1990). Löschenberger et al. (1993) reported that 97% of the anther culture-derived regenerants of tetraploid wheat were albino and only 3% green. In the present experiment, 76.5% of the regenerants developed chlorophyll, making the

green to albino ratio somewhat encouraging.

5.2.2.1. Growth Condition and Yield of Green Regenerants

We found that field-grown donor plants yielded relatively more green plants, presumably due to more protoplasts present in anthers because the plants were grown in the field were healthy and vigorous. Similar results were obtained by Ouyang et al. (1987), Bjørnstad et al. (1989), and Foroughi-Wehr and Zeller (1990). Several studies have shown that donor-plant growth condition strongly influences the yield of pollen plantlets in bread wheat (Ouyang et al., 1987; Simmonds, 1989; Orshinsky and Sadasivaiah, 1997; Holme et al., 1999). The expression of genes for chlorophyll development is influenced by environmental factors, such as growth conditions of the anther-donor plants (Bjørnstad et al., 1989) and cold pretreatment of anthers (Powell, 1988; Konzak and Zhou, 1991).

5.2.2.2. Anther-Culture Media and Albinism

It is difficult to explain the wide-spread occurrence of albino plantlets. The constituents of the basal medium serve as important factors in eliciting successful androgenesis. Media composition in anther cultures has been extensively studied over the years. The available literature does not suggest any one culture medium that could be applicable to all systems. The media requirements vary from genotype to genotype. Media formulations and culture conditions may also contribute to the production of albino plantlets. Accumulations of lactic acid and other organic compounds or metabolic by-products during culture may damage the organelles in primordial cells and result in the production of albino plantlets (Kao, 1981). It is also possible that the chemical composition of the medium, coupled with culture conditions, causes mutation of the gene(s) that control chlorophyll development. The recovery of chimeric plantlets (Figure 4.5 I) shows that such a presumed gene mutation occurred during the formation of tiller primordia, so the mutant and the normal tiller primordia responded differently to chlorophyll development.

A strong media effect on anther response and callus production was observed

in the present study. Anther response was better on BAD-1 and BAC-1 than on BAD-3 and M-42. BAD-1 also gave the highest callus production and M-42 the lowest. M-42 gave the highest green plant regeneration, but differences among the four media were not significant for green plant regeneration. The culture media formulation is reported to be a major factor for anther response and induction of green plantlets from microspores (Fadel and Wenzel, 1990; Orshinsky et al., 1990; Last and Brettel, 1990; Kao, 1993; Trottier et al., 1993; Cho and Kasha, 1995; Hu, 1997).

In the present study, the regeneration of more green plants than earlier reports might be due to use of liquid media supplemented with ficoll. Even though there was no significant difference, it is important to note that a higher green plant regeneration occurred on M-42, which contains a higher ficoll content than the other media used. The positive effect of liquid media with ficoll was reported by several researchers (Kao, 1981; Zhou et al., 1991; Trottier et al., 1993). According to Zhou and Konzak (1989), more than 85% of the calli produced in anther culture of wheat were floating on the surface of the ficoll-supplemented medium, compared with only 39 to 57% in medium lacking ficoll, which resulted in a doubling of the plant regeneration. The ratio of green to albino plantlets was also improved by the ficoll supplement, which was further confirmed by Lashermes (1992).

5.3. Chromosomal Changes

5.3.1. Induced Polyploidy

The green regenerants of durum wheat from the present study showed a wide variation in chromosome number, mostly resulting in multiplication of chromosome number. The ploidy level of the regenerants varied from $2n = 28$ to 70, although several aneuploids were also observed. Changes in chromosome number and structure are the most frequently observed types of genetic variation and have been reported in microspore cultures and regenerated plants (D'Amato, 1985). In the anther culture of cereals, most of the regenerants are diploid, and the rest are either haploid or tetraploid (Wenzel and Foroughi-Wehr, 1984).

Polyploidy is the most frequently observed chromosomal abnormality, although aneuploidy has also been observed frequently in cell cultures (Evans, 1989). The most common form of polyploidy is a repeated doubling of the basic set of chromosomes (*2n*, *4n*, *8n*, etc.), which can occur by endoreduplication (in which the centromeres of sister chromatids do not separate after DNA synthesis), and/or endomitosis (chromatid replication without separation, resulting in polytene chromosomes), or by fusion of the vegetative and generative nuclei present within the same microspores (Sunderland, 1977). McComb (1978) also stated that irregular meiosis, endomitosis, spindle fusion, nuclear fusion, and endoreduplication are the main phenomena that contribute to polyploidy. Aneuploids have also been reported by other researchers (e.g., Karp and Maddock, 1984; Xingzhi and Han, 1985).

Several factors may affect the ploidy levels, such as cold pretreatment (Amssa et al., 1980; Chen and Beversdorf, 1992), pollen (microspore) stage, growth regulators or epigenetic factors (Wenzel and Foroughi-Wehr, 1984), length of the culture time (McCoy et al., 1982; Xi et al., 1986; Henry et al., 1996), or sugar content in culture media (Navarro-Alvarez et al., 1994).

It has been reported that increasing the number of chromosome sets can result in increases in plant size, but can also have a detrimental effect on growth when the gene dosage is too high; polyploidy and aneuploidy also result in decreased fertility (Karp and Bright, 1985; Evans 1989). In our study, most of the polyploid and aneuploid regenerants were smaller than the parent plants (Figure 4.6 C) and were not fertile, whereas spontaneous DHs were fertile and set seed.

5.3.2. Preferential Multiplication of B-Genome Chromosomes

An interesting observation made in the present study was the preferential multiplication of B-genome chromosomes. Fluorescent genomic *in situ* hybridization of polyploid cells revealed that B-genome chromosomes were present in disproportionately higher numbers than A-genome chromosomes. Thus, the cells shown in Figures 4.11 A and B each contain 14 A-genome chromosomes and 21 B-genome chromosomes. The preferential multiplication of B-genome chromosomes was further evidenced by the presence of intragenomic multivalents within the B-

genome. The preponderance of B-genome chromosomes may be related to their predominantly heterochromatic nature. Heterochromatin is known to replicate later than euchromatin. That is why B chromosomes (accessory or supernumerary chromosomes) undergo nondisjunction, the mechanism by which they multiply (Jones and Rees, 1982). However, Winfield et al. (1995) reported that chromosomes of the B-genome were most often lost or involved in rearrangements, with breakpoints located at or near the heterochromatic blocks in microspore-, anther-, and immature embryo-derived regenerants. Chromosomal changes involving multiplication of entire genomes or some chromosomes have been reported in callus cultures of wheat (Chen and Chen, 1980; Wang and Hu, 1985; Henry et al., 1996; Stober and Hess, 1997).

Some of the pentaploid durum regenerants ($2n = 5x = 35$; AABBB) we obtained looked more like bread wheat. Figure 4.6 D, for example, shows a pentaploid ($2n = 35$) plant with spikes similar to those of bread wheat. It is likely that an additional dose of the B-genome chromosomes "compensated" for the absence of the D-genome chromosomes in durum wheat. This may perhaps be called compensation at the genomic level. If this phenomenon does indeed occur, it is analogous to nullisomic-tetrasomic compensation observed by Sears (1966) at the chromosomal level.

5.3.3. Ploidy Level and Chlorophyll Development

There appeared to be a relationship between polyploidy and the development of chlorophyll. The frequency of green plants increased with the increase in level of ploidy. Haploid plantlets with 14 chromosomes were all albino. Plants with 28 chromosomes were either albino or green. However, it is interesting that plants with more than 28 chromosomes were all green. It appears as if the development of chlorophyll was ploidy-dependent. It is likely that multiplication of both nuclear and cytoplasmic factors in the polyploid regenerants were responsible for chlorophyll production. A ploidy-dependent gene expression has been elegantly demonstrated by Galitski et al. (1999).

5.4. Structural Changes in Chromosomes and Gene Mutations

We observed several different types of structural abnormalities of chromosomes including telocentrics, dicentrics, and terminal or interstitial translocations. Robertsonian translocations, lightly stained regions, and centric or acentric fragments were also observed (Doğramacı-Altuntepe et al., 1999). Several structural aberrations such as duplications, deletions, inversions, translocations and interchanges have been reported in the chromosome complement of microspore-derived plants (Kudirka et al., 1983; Chu et al., 1985; De Buyser and Henry, 1986; McCoy et al., 1982; Karp and Maddock, 1984; Lee and Phillips, 1988).

Chromosomes occurring as univalents at meiosis undergo misdivision (breakage at the centromere), resulting in two telocentrics. The telocentrics have a tendency to reunite. Union or fusion of arms of different chromosomes at the centromere would result in Robertsonian translocations. Events leading to chromosome breakage and, in some cases, subsequent exchanges or fusions of broken ends may therefore be of fundamental importance. Desirable Robertsonians may arise by the union of two arms from different chromosomes with desirable genes.

Genetic changes such as chromosomal aberrations and gene mutations are known to occur during *in vitro* cultures of cells and tissues in a wide range of plant species (D'Amato, 1977; Bayliss, 1980). Genetic alterations in tissue culture occur at much higher frequency than would be expected through spontaneous mutation (Evans and Sharp, 1986; Larkin, 1987). However, the mechanism for induction of such variations is still unknown. It is suggested that the variations originate from culture medium and conditions, as well as polyploidy and genetic constitution of the initial explants. Among the components in the medium, synthetic auxins such as 2,4-D may be the most likely mutagens.

Studies on the genetics and cytology of variants have revealed that changes occur in chromosome number and structure, DNA content, gene loci, plastid DNA, and also epigenetically (Karp and Bright, 1985). Attempts to identify the origins of genetic variation in cell cultures are complicated by the possibility that the various changes may arise in different ways and vary between species and genotypes

(Ziauddin and Kasha, 1990). It has been suggested that the genetic variation observed in tissue culture may result from both pre-existing variation in the explant donor tissue and variation induced during the tissue culture process itself (Evans et al., 1984; Morrison and Evans, 1987; Evans, 1989).

5.4.1. Chromatid Breaks and Exchanges

In the present study, chromatid breaks and exchanges were observed. Fluorescent GISH analysis showed the occurrence of non-sister chromatid exchanges involving the A- and B- genomes. It is difficult to say whether sister chromatid exchanges (SCEs) occurred because they would not be detected by GISH. The 2,4-D (2-8 mgL^{-1}) in the media used may have contributed to chromatid exchanges observed in this study. The potential for genetic damage from the widely used hormonal herbicide 2,4-D has been demonstrated by Turkula and Jalal (1985). They showed the mutagenic effect of 2,4-D (50-250 mgL^{-1}) as reflected by the rates of SCEs in cultured human lymphocytes. Several researchers have reported the occurrence of SCEs in plant cell cultures. Murata (1989) reported the effect of 2,4-D (2.0 mgL^{-1}) on the induction of SCEs in cultured cells of wheat: the mean number of SCEs per cell was 15.2. Pijnacker and Ferwerda (1994) also showed the effect of 2,4-D and sucrose on SCEs in cell cultures of *T. aestivum*, *T. durum*, *T. dicoccum*, and *T. monococcum*. The tetraploids, *T. dicoccum* and *T. durum*, had relatively high mean numbers of SCEs.

5.5. Gametoclonal / Somaclonal Variation

Anther cultures leading to callus production often generate genetic variation called gametoclonal variation (Morrison and Evans, 1987), which describes phenotypically variant plants regenerated from gametophytic cells. The anther culture-derived durum regenerants obtained in the present study showed several phenotypic abnormalities. Variations in growth habits and morphology became obvious in mature plants. This gametoclonal/somaclonal variation was presumably caused by chromosomal aberrations and other genetic changes caused by culture

conditions. Marburger and Jauhar (1989) also suggested that *in vitro* induced chromosomal aberrations during the regeneration process may have contributed to gametoclonal variation.

Although the characteristics of the variability found in microspore cultures are often definable, the actual causes of the variation in microspore cultures and among the regenerated plants are still unclear. A number of mechanisms have been implicated. These can be related to the microspore culture process, such as culture-induced chromosome breakage associated with regions of heterochromatin, which may lead to the activation of transposable elements, or changes in the methylation induced by the "stress" of the culture phase (Logue, 1996). Variations may also arise from chance events, so that even when conditions are identical, repeat experiments may give inconsistent results (Karp, 1989).

Conclusion and Perspectives

The results obtained from this study are of both basic and applied value. Several numerical and structural changes in chromosomes have been recorded, some for the first time. Thus, the preferential multiplication of B-genome chromosomes in cultures offers a challenge to cytogeneticists. The resemblance of pentaploid ($2n = 5x = 35$; AABBB) durum wheat regenerants to hexaploid bread wheat would suggest "compensation" at the genomic level. This may stimulate further research along these lines.

The structural changes in chromosomes may constitute the basis of gametoclonal/somaclonal variation. Whatever the causes of this variation, it may prove to be of significance from the breeding standpoint (Brar and Jain, 1998). If the causes of gametoclonal variation could be understood and manipulated it would provide the breeder with another valuable tool for generating the variability needed in breeding programs (Logue, 1996). Potential applications of somaclonal variations have been proposed as a supplementary tool to well-established breeding approaches such as cross-breeding and mutation breeding for crop improvement. A somaclonal variant wheat, 'Hezu-8', has been released for commercial production in China (Gao et al., 1992). However, the frequency and type of somaclonal variations in a number

of plant species, particularly in cereal crops, are low and largely undesirable (Cheng et al., 1992).

On the other hand, at least 20 cultivars of wheat have been produced in China using tissue culture techniques (Hu, 1991, 1992, 1997). These cultivars, with superior agronomic traits (high yields and wide adaptation), are reported to be cultivated on more than one million ha (Hu, 1997). Lu et al. (2000) continue to use somatic tissue culture and anther culture to induce and stabilize variation to improve wheat breeding efficiency in China. As stated earlier, haploids and DHs have great importance for breeding (Khush and Virmani, 1996), genetic analysis (Hu, 1997), genome mapping (Zivy et al., 1992), and gene transformation (Jahne et al., 1994; Jauhar and Chibbar, 1999). Thus, the development of an efficient anther-culture protocol is important and may have significant implications in durum wheat improvement. To standardize the anther culture technique for durum wheat new media formulations can be examined in future experiments using the successful cultivars for green plant regeneration from this study.

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