The exploit of cereal embryo structure for productive reasons by in vitro techniques

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Abstract. There are two main sides of our works exploiting embryo structure in durum wheat and some other cereals. First is haploid (or doubled haploid) embryo production using anther or microspore culture or intergeneric crosses, to ameliorate desirable characters genetically homozygote. Secondly, to develop convenient embryo culture techniques in order to be stored and cultivated longer time of genotypes without being alien pollination etc. in field conditions. For that reason, two different auxin and also their combination with kinetin were used for mature embryos of wheat genotypes (hexaploid and tetraploid), to understand efficient dose for calli production and plant regeneration in plant tissue culture. Modified MS media were used adding a single dose of arabinogalactan protein (AGP) and without adding for regeneration. In further step of this study, most efficient auxin+kinetin combination which is determined previous research, it was used in the same modified MS medium to produce calli production and plant regeneration in three different genotypes (hexaploid and tetraploid wheat and diploid barley). Data were calculated in five different developmental stages of treatments. All statistical analysis of data were performed and means were compared with Duncan’s test. Genetics and morphological effects of AGP on genotypes were discussed with the results of variance analysis. Simple correlation coefficient (r) was calculated base on the main values of replications.

1. Introduction
An embryo is an useful organisation to manipulate with various techniques for agricultural or biological objectives particularly in angiosperms. It can give sufficient answers in different developmental stages and parts which are related with either genetical components or anatomical structures. Monocotyl plants (mainly cereals) carry a tremendous capacity for showing of these manipulations in further generations, such as variation in genetics, differences in anatomy or other desirable characters in agriculture, because of their numerical abundancy in morphology. A mature embryo as a part of a seed besides endosperm and seed coat creates fertile individual for further generation of its species (figure 1).

A seed is a very valuable source that can be belonging to a wild type, landrace or a cultivar improved by selection with breeding methods. Adaptation, yield and quality or related values are features expressed by gene(s) which are found as genetic materials into seed. The long term usage of these features is necessary for humankind in production and nutrition. However, its productivity reduces with many uses, environmental factors or foreign pollinations etc., during the cultivation. There are two main sides of our works generally.
Figure 1. A mature diploid bread wheat embryo (Triticum aestivum L.,) (left). A haploid embryo produced using wheat x maize crosses (middle) and its anatomical structure (right) [1]. I. radicle and coleorhizae, II. plumula and leaf primordium, III. scutellum, IV. stem (at right).

- haploid (or doubled haploid) embryo production using anther or microspore culture or intergenic crosses, to ameliorate desirable characters as genetically homozygote to long term use,
- to exploit of mature embryo structure for protection of phenotypic characters without losing genes in genotypes. Thus, ameliorated genotypes can be stored or used in agronomy for longer time.

Here, the point of view is to contribute to own values of nature for the environment rather than industrial approach, in other words, the prevail subject is the protection of varieties for the beneficial of small size farming, using tissue culture technique facilities. Because there are many varieties improved by genetical and breeding methods, particularly in crop species all over the world, up to now. These genetical sources, landraces and old or obselet cultivars can be used again for further generations without sharing huge natural resources and budgets for new varieties (figure 2).

Figure 2. Revealing of variation as different genotypes from old/obselet durum wheat landraces after making homozygote of alleles by doubling of chromosomes using intergeneric crosses or embryo rescue. Grains of doubled haploid (DH) genotypes derived from T.durum cv. Berkmen 469 (left) and Kunduru 1149 (right) [4].

In tissue culture techniques, the main function is to product abundant and healthy productive embryos or embryoid calli in induction and differentiation for regeneration on culture media, respectively. Therefore supportive growth regulators, proteins or other physical factors will be able to provide a rich culture medium for plant materials (explants). For that reason, we considered two important details in mature embryo studies using two ploidy levels of wheat and also a diploid barley. As first, the effectiveness of growth regulators (auxin and cytokinin) for induction embryoid-calli was searched in wheat [2]. Secondly, the effects of various amounts of Arabinogalactan-Proteins (AGPs),
which are described extracellular proteoglycans in most cases, were used for high regeneration capacity in hexaploid and tetraploid wheat and also a diploid barley genotypes [3]. In this presentation the importance of genetic and morphological effects of AGPs in differentiation were discussed in various ploidy levels of these genotypes, exploiting mature embryo structure.

2. Materials and methods

2.1. Plant materials
In our researches, mainly durum wheat has been used as plant material. Wheat is one of the important crop plant economically and has a long period of evolutionary history. There are two very important species widely used by humankind, *Triticum aestivum* (hexaploid, bread wheat) and *T. durum* (tetraploid, durum wheat). Besides these, some other cereals have been worked in our tissue culture studies in anther culture technique and intergeneric crosses for germplasm evaluation and for also comparison of haploid-diploid embryo structure [1,4,5].

In mature embryo culture studies, three different ploidy levels of cereals, bread wheat (hexaploid-AABBDD), *T. aestivum* cv. Ikiçe-96 (2n=6x=42), durum wheat (tetraploid-AABB) *T.durum* Desf. cv. Mirzabey (2n=4x=28), and barley (diploid-AB) *H.vulgare* L. cv. Tokak 157/37 (2n=2x=14) were chosen between Anatolian cultivars. Seeds of cultivars were obtained from the Field Crops Central Research Institute, Ministry of Agriculture and Rural Affairs in Ankara, Turkey [2,3].

2.2. Hormones and Arabinogalactan Proteins (AGPs)
Hormones: Four different hormone combinations were treated by adding to MS (modified) medium [6] supplemented with 20 g L\(^{-1}\) sucrose and 7 g L\(^{-1}\) agar, to understand the effects of calli production in each of treatment groups. Auxins (Dicamba and 2,4-D) 5mg L\(^{-1}\) and also their combinations with cytokinin (Kinetin) 1 mg L\(^{-1}\) were prepared to searched in the first treatment group and among them, Dicamba+Kinetin (5 mgL\(^{-1}\)+1 mgL\(^{-1}\)) hormones were given the best result [2]. That determined combination was used for calli production in the second step of study [3].

AGPs: AGPs have been shown to be expressed throughout the plant kingdom and have been considered to have important roles in plant growth and developments. The majority of the AGP has a protein content less than 10%, and contains more than 90% carbohydrate. They are water soluble non-starch pentosan group glycoproteins and can be found many parts of a plant, in vacuoles [7] particularly cell-wall structure [8]. Wilkinson et al (2013) reported that numerous AGP sequence types were identified in Poaceae family members in *Agropyron mongolicum, Secale cereale, Oryza sativa* subsp. *japonica* and *Sorghum bicolor*. Since the AGPs are rich in hydroxyproline they are classified as hydroxyproline-rich glycoproteins (HRGPs) [9]. Coskun et al (2010) found that AGP increased plant regeneration in wheat genotypes when they were added 10 ml L\(^{-1}\) into MS medium [2]. However, at the following study, they determined that 5 mg L\(^{-1}\) of AGP is the best quantitiy for a suitable regeneration system between six different AGP concentrations (0,2,5,7,10,12 mg L\(^{-1}\)), in three ploidy levels of cereals including mature embryos of bread and durum wheat and diploid barley [3].

2.3. Data analysis
Data were analysed as follows: Callus induction efficiency = (Number of calli/Number of mature embryo) x 100. Plant regeneration efficiency = (Number of plant regenerant/Number of mature embryo) x100. Culture efficiency = (Callus induction efficiency/Plant regeneration efficiency) x 100. Weight of callus = Each callus was weighed on their 14th day of culture. Measurement of callus = Each callus was measured on their 14th day of culture. The length of root, stem and total plant height= Each plant was measured after 6 weeks of culture.

Statistics: Each of petri plate containing 20 embryos was considered as single of replication and treatment was conducted according to randomised complete design with four replications for each AGP regeneration treatment. SPSS 15.0 software programme was used for statistical analysis and means were compared with Duncan’s Test in each means of application of hormones and means.
between culture media groups. Simple correlation (r) were calculated based on the mean values of replications [10].

3. Results and discussion

The results of the influential effects of some of hormone treatments on callus production derived from mature wheat embryos, and, efficient plant regeneration with AGPs on various ploidy levels of cereals have been discussed in Coskun et al (2010, 2013) [2, 3]. Analysed data (parameters) were performed with variance analysis and means were compared with Duncan’s test in each of two researches.

Callus weight was statistically different between genotypes (P<0.01) (table 1). The lowest weight one was found in Ikizce-96 (79.91 mg) while its callus induction (93.75%) was the highest (table 1). Tokak 157/37 with the smallest number of genome (AB) produced the weightiest (179.95 mg) and largest callus (6.18 mm) (table 1). In contrast diploid Tokak 157/37 produced less regenerant plants than other genotypes (tetraploid and hexaploid) [3]. Savaskan et al (1999) used Tokak 157/37 in androgenesis to produce haploid regenerant plants, and they observed that this genotype produced less regenerant plants than other diploid barley genotypes (Cumhuriyet-50, Anadolu and Obruk) although it produced much more embryoid calli than the others [5]. Bi and Wang (2008) compared diploid and tetraploid wheat genotypes for capacity of totipotency and mentioned that tetraploid wheats produced more calli and regenerant plants than diploids [11]. Callus induction didn’t show differences statistically between either two hexaploid and tetraploid wheat genotypes [2] or wheats and diploid barley [3] when Dicamba +Kinetin (5 mg L\(^{-1}\) + 1 mg L\(^{-1}\)) combination was used in MS induction medium.

Table 1. Variance analysis and means of calli procedures of three genotypes.

| Genotype     | Genome  | Callus induction (%) | Weight of callus (mg) | Diameter of callus (mm) |
|--------------|---------|----------------------|-----------------------|------------------------|
| Ikizce-96   | AABBDD  | 93.75 ± 3.53 a       | 79.91 ± 3.23 a        | 4.56 ± 0.25 a          |
| Mirzabey    | AABB    | 93.50 ± 3.57 a       | 83.84 ± 1.79 b        | 4.69 ± 0.11 a          |
| Tokak 157/37| AB      | 91.25 ± 2.31 a       | 179.95 ± 3.13 c       | 6.18 ± 0.25 b          |
| F-value     |         |                      | n.s.                  | **                     |

*Entries within column followed by the same letter are not significantly different (P<0.05). n.s. = non significant, * P<0.05, ** P< 0.01, [3].

3.1. Genetic effects of AGPs on differentiation

Researchers stated that the wheat Gsp-1 gene resulted in reduced contents both AGP and grain softness protein (GSP-1) in mature wheat grains confirming that these components are encoded by the same gene [7,12]. In earlier studies, other researchers had already reported that Gsp-1 genes are localised on the 5A1, 5B1 and 5DS chromosomes of wheat and linked to the grain hardness lokus (<I>Ha</I>) on 5D [13,14]. In that point, it is possible to understand that the degreasing of gene effects on calli differentiation according to the genom numbers of genotypes were found significantly different (table 2). In other words, differences between regeneration capacity (%) and also culture efficiency (%) of genotypes were important (P<0.01). Similarly the variance for AGP treatments for these two parameters were found important as (P<0.05) and (P<0.01) respectively (table 2). Genotype x AGP interactions on three genotypes (ploidy levels) were found significant (P<0.01) at both parameters related with differentiation, regeneration capacity (%) and culture efficiency (%) (table 2).

3.2. Morphogenesis of AGPs for plant development

Also hexaploid genotype (Ikizce-96) gave the higher value for root and stem length and total plant heigth as 12.46, 19.00 and 31.46 cm in plant regeneration using MS+5 mg L\(^{-1}\) AGP while diploid Tokak 157/37 produced the lowest values in the same group of regeneration medium, 10.39, 13.39 and 23.78 cm, respectively [3]. The length of root and stem and also total plant heigth were measured in all regenerant plants after six weeks [3]. In genotypes, morphological values of regenerant plants (root and stem length and total plant height) improved in existence of AGP (P<0.01) (table 2). At the organ
level, AGPs are found in leaves, stems roots, floral parts and seeds [15,16]. For that reason, it is possible to expected that genotype x AGP interaction in morphogenesis (plant growth and development) was found significantly different (table 2).

### Table 2. Analysis of variance for AGP treatments on three different genotypes.

| Variation Sources | df | Root Length (cm) | Stem Length (cm) | Plant Height (cm) | Regeneration Capacity (%) | Culture Efficiency (%) |
|-------------------|----|------------------|------------------|-------------------|--------------------------|------------------------|
| Genotype          | 2  | 8.196**          | 11.275**         | 13.269**          | 21.973**                 | 16.842**               |
| AGP               | 5  | 5.223**          | 3.935**          | 4.286**           | 3.584*                   | 4.869**                |
| Gen. x AGP        | 10 | 27.087**         | 28.457**         | 29.895**          | 66.768**                 | 67.566**               |

All characters correlated to each other on positive side when AGP added 5 mg L\(^{-1}\) to MS medium for embryo culture and simple correlation between culture efficiency and regeneration capacity was found important (P<0.01) (table 3). Also, either culture efficiency or regeneration capacity were correlated with root length significantly important (P<0.01). Correlation between culture efficiency and root length was found as r = 0.9999** (table 3) and the effect of auxin (Dicamba) can be resulted in to this value. Other important auxins naphlol acetic acid (NAA) and indol acetic acid (IAA) had been used in a previous barley anther culture technique for remedy to rootlessness at the regenerant plants which is an important problem for anther culture [5]. In that study, high amount of NAA or IAA (10 mg L\(^{-1}\)) had been added to regeneration medium supplemented with 4 mgL\(^{-1}\) charcoal to promote rooting [5]. On the other hand, culture efficiency and regeneration capacity didn’t correlate with stem length importantly (table 3). The physiological events of hormones and proteins might have been prevailed more than genetical effects in stem development.

### Table 3. Simple correlation coefficients (r) between the parameters (characters) of three ploidy levels of genotypes according to the results of 5 mg L\(^{-1}\) AGP applications.

| Parameters of treatments                                      | r   |
|--------------------------------------------------------------|-----|
| Culture efficiency – regeneration capacity                   | 0.9988** |
| Culture efficiency - root length                             | 0.9999** |
| Culture efficiency – stem length                             | 0.9500*  |
| Stem length - regeneration capacity                          | 0.9646*  |
| Root length – regeneration capacity                          | 0.9988** |

Significant at the 0.01**, 0.05* probability levels, non significant*n.s.*

### 4. Conclusion
- A mature embryo is a vital part of dry dormant seed in plants. Biological and biotechnical manipulations in cereal embryos are being promising procedures for many targets related with basic researches and agronomy [1,4].
- Dicamba is a stronger auxin in wheats particularly combination with Kinetin than 2,4-D. Dicamba+Kinetin (5 mg L\(^{-1}\) + 1 mg L\(^{-1}\)) enriched the induction medium and supported calli production significantly [2].
- In various ploidy levels of genotypes, modified MS with 5 mg L\(^{-1}\) AGP was given best results in all parameters related with regeneration and morphogenesis. Also, 2 mg L\(^{-1}\) of AGP to regeneration medium was provided succesfull results [3]. It may be an economical amount for larger practises and demonstrations.
- According to the results, AGP can be used as a supportive agent in mature embryo culture in cereals. Because it intreated with genotypes in respect of increasing regeneration and morphogenesis significantly (P<0.01) (table 2). However, correlation between regeneration
capacity with stem length didn’t show important relationship although it correlated with root length significantly (table 3). This can be interpreted that genetic effects of AGP changed in different parts of plants.

References
[1] Ozkara A and Savaskan C 2014 Wheat maize crosses for haploid embryo production and comparison of haploid-diploid embryo structure Res. J. Biotech. 9 32-7
[2] Coskun Y, Duran R E and Savaskan C 2011 Influential effects of arabinogalactan-proteins on plant regeneration using calli derived from wheat mature embryos Afric. J. Agric. Res. 5 2439-45
[3] Coskun Y, Duran R E, Savaskan C, Demirci T and Hakan M T 2013 Efficient plant regeneration with arabinogalactan-proteins on various ploidy levels of cereals J. Integr. Agric. 12 420-5
[4] Savaskan C, Akinci C, Donmez E, Keser M and Yalvac K 2003 The relationships between the grain characters of Anatolian durum wheat genotypes 10th Inte. Wheat Genetics Symp. (Paestum, Italy) 1 81-4
[5] Savaskan C, Szarejko I and Toker M C 1999 Callus production and plant regeneration from anther culture of some Turkish barley cultivars T. J. Bot. 23 359-65
[6] Murashige T and Skoog F 1962 A revised medium for rapid growth and bioassays with tobacco tissue culture Physiol. Plant 15 473-97
[7] Wilkinson M D, Castells-Brooke N and Shewry P R 2013 Diversity of sequences encoded by the Gsp-1 genes in wheat and other grass species J. Cereal Sci. 57 1-9
[8] Minorsky P V 2002 The Wall becomes surmountable Plant Physiol. 128 345-53.
[9] Kreuger M and van Holst G J 1996 Arabinogalactan proteins and plant differentiation Plant Mol. Biol. 30 1077-86
[10] Freed R D 1991 MSTATC: Microcomputer statistical program. experimental design, data management and data analysis (Michigan, USA: Crop and Soil Department, Mich. Michigan State Univ.)
[11] Bi R and Wang H 2008 Primary studies on tissue culture from mature embryos in diploid and tetraploid wheat Front. Agric. China 2 262-5
[12] Wilkinson M D, et al 2017 The Gsp-1 genes encode the 1 wheat arabinogalactan peptide J. Cereal Sci. 74, 155-64
[13] Jolly C J, Glenn G M and Rahman R 1996 GSP-1 genes are linked to the grain hardness locus (<i>Ha</i>) on wheat chromosome 5D Proc. of the National Academy of Sciences (USA) 93 2408-13 (in Catalogue of Gene Symbols for Wheat. 10.IWGS, paestum, Italy 2003 Produced by McIntosh R A, Yamazaki Y, Devos K M, Rogers J and Appels R)
[14] Turner M, Muhai Y, Leroy B, Chatel B, Appels R and Rahman S 1999 The <i>Ha</i> locus of wheat: Identification of a polymorphic region for tracing grain hardness in crosses Genome 42 1242-8 (in Catalogue of Gene Symbols for Wheat. 10.IWGS, paestum, Italy 2003 Produced by McIntosh R A, Yamazaki Y, Devos K M, Rogers J and Appels R)
[15] Fincher G B, Stone B A and Clarke A E 1983 Arabinogalactan proteins: structure, biosynthesis and function Ann. Rev. Plant Physi. 34 47-70
[16] Ma H and Zhao J 2010 Genome-wide identification, classification, and expression analysis of the arabinogalactan protein gene family in rice (Oryza sativa L.) J Exp Bot 61 2647-68