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Araştırma Makalesi/Research Article

Response of Current Winter Wheat Cultivars Grown in Turkey to Immature Embryo Culture

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ABSTRACT : Selecting the explant genotypes is crucial step in *in-vitro* culture and *Agrobacterium*-mediated transformation system due to its host range specificity. Poor tissue culture performance is restricting the number of wheat genotypes that can be stably transformed. Response of fourteen cultivars of winter wheat (*Triticum aestivum* L.) to immature embryo culture, callus production and plant regeneration was evaluated. A significant ($p < 0.05$) genotypic difference in callus induction frequency was observed ranging between 45% (cv. Sürak) and 99.54% (cv. Bobwhite) of the cultured explants. Plant regeneration through somatic embryogenesis was observed from calli of all 15 cultivars after a 4-week callus induction period. Genotypes with the highest number of plantles per cultured embryo were Sivas-111/33 (8.66), Kıraç-66 (7.36), Haymana 79 (6.77) and Conkesme (6.56). Four winter wheat genotypes, 'Sivas-111/33, Kirac-66, Haymana 79, and Conkesme', have been selected for initial *Agrobacterium*-mediated transformation experiments due to their high regeneration potentials.

Key words: Winter wheat, callus formation, plant regeneration, immature embryos

Türkiye'de Yetiştirilen Bazi Kişlik Buğday Çeştilerinin Olgunlaşmamiş Embriyo Kültürüne Verdiklerin Cevaplarin Belirlenmesi

ÖZET : *Agrobacterium* kaynaklı gen aktarımı ve *in vitro* kültür çalışmaları için kullanılacak eksplant genotipin seçimi önemli bir adımdır. Düşük doku kültürü performansı, stabil gen aktarılabilecek buğday genotiplerin sayısını sınırlamaktadır. 14 kışlık buğday çeşitinin olgunlaşmamış embriyo kültürü, kalus üretimi ve bitki rejenerasyonuna tepkisi değerlendirilmiştir. Kültüre alınaneksplantların kallus oluşum frekansı %45 (cv. Sürak) ve % 99.54 (cv. Bobwhite) arasındaki oranlarda gözlenmiş ve genotipik farklılık önemli olmuştur. Somatik embriyogenesis aracılığıyla bitki rejnerasyonu 4 haftalık kallus oluşum periyodundan sonra tüm çeşitlerin kalluslarında görülmüştür. Kültüre alınan embriyo başına en yüksek bitkicik sayısı Sivas-111/33 (8.66), Kıraç-66 (7.36), Haymana 79 (6.77) and Conkesme 79 (6.56) çeşitlerinde meydana gelmiştir. Dört kışlık buğday genotipi (Sivas-111/33, Kirac-66, Haymana 79, and Conkesme) yüksek rejenerasyon potansiyeline sahip olmaları nedeniyle *Agrobacterium* aracılığıyla gen aktarım çalışmalarında kullanmak için seçilmiştir.

Anahtar Kelimeler :

INTRODUCTION

One of the critical aspects of successful plant transformation *in vitro* is the ability to form tissues suitable for transformation and to regenerate whole plants from the transformed tissue. Wheat (*Triticum aestivum* L.) immature embryos have been determined to be most efficient tissue source to regenerate whole plants in large numbers (Ahloowalia 1982, Sears and Deckard 1982, Ozias-Akins and Vasil 1982) and are used extensively in genetic transformation studies of wheat (Weeks et al. 1993, Cheng et al. 1997, Haliloglu and Baenziger 2003).

Significant contributions can be made to wheat improvement, when the potential offered by genetic transformation is combined with the success of conventional breeding techniques. The first step coupling transformation with conventional breeding is the identification of elite genotypes suitable for transformation that can be used as a parent. Transformation requires the development of an efficient callus initiation, transformation, regeneration system, and ideally, for wheat breeding, parents should be of the same growth habit and market class, and have similar adaptation genes. Many studies (Sears and Deckard 1982, Mathias and Simpson 1986, Haliloglu et al. 2005) have reported that the explant genotype plays an important role in callus induction, maintenance and regeneration from immature embryos.

The purpose of our study was to identify responsive genotypes for immature embryo culture by screening fourteen current winter wheat genotypes grown in Turkey. From our study, successful genotypes were identified and will be used for *Agrobacterium* mediated genetic transformation experiments.

MATERIALS AND METHODS

Plant materials

Fourteen winter wheat (*Triticum aestivum* L.) cultivars obtained from the various Seed and Plant Improvement Institutes, Turkey, (Table 1). A spring wheat "Bobwhite" was used as a control. Cultivars were grown in Experimental Field of Ataturk University. Same number of spikes were harvested

and used in each treatment in the following experiments.

Immature embryo culture

Immature caryopses were collected from the plants 14 days after anthesis. Immature embryos were dissected aseptically and cultured on a semisolid callus initiation medium which was a CM4 medium (Zhou et al., 1995) with 100 mg L^{-1} ascorbic acid (CM4C). Full strength (the original amounts) of MS salts (Murashige and Skoog, 1962) was used in the CM4C medium. Explants were cultured at 25 °C in the dark, and 30 days later, the callus induction, callus weight and somatic embryo formation was measured. Callus development during induction and initiation was periodically monitored.

Regeneration response was compared after a 4 or 8-week callus induction period including no or one subcultures respectively. For regeneration, calli were transferred onto regeneration medium (full strength MS salt and vitamins without 2,4-D) with 40 g/l maltose, solidified with 0.25% Gelrite and the pH adjusted to 5.8 prior to autoclaving. Cultures were kept under fluorescent light with 15000 lx and 16 h/8 h light/dark cycle. Twenty days after transfer to regeneration conditions the number of shootlets ca. 1.5 cm in length or longer was recorded. Shootlets of this size normally develop into mature plants if separated and propagated individually.

Statistical analysis

Experiment was carried out using Completely Randomized Design (CRD) with 4 replications of 25 embryos per plate. The callus formation percentage was calculated as the number of embryos forming callus as a percentage of cultured embryos. The percentage of embryogenic calli was evaluated as the number of calli producing somatic embryos as a percentage of the total number of calli induced. The number of plantlets per cultured embryo resulted from the total number of regenerated plantlets divided by the number of cultured embryos. Analysis of variance and the Waller-Duncan K-ratio *t*-test (Waller and Duncan, 1969) were used to compare the means. SAS/PC statistical program was used for all computations (SAS Institute Inc. 1996).

RESULTS

Immature embryos of 14 current Turkish winter wheats and the tissue culture model cultivar Bobwhite were produced from four plots of completely randomized donor plants. Callus could be induced from immature embryos of all of the evaluated 15 winter wheats. A significant ($p<0.05$) genotypic difference in callus induction frequency was observed ranging between 45% (cv. Sürak) and 99.54% (cv. Bobwhite). Winter wheat genotypes, Tir, Sabalan, Hawk and Sivas-111/13 followed the Bobwhite; they were equal and highly responsive genotypes to callus formation (Table 1). On the other hand, genotype with the lowest callus formation was Sürak (45%). The genotypic difference in callus regeneration frequency (as a percentage of embryogenic calli induced) and in the number of regenerated plantlets per cultured explant was highly significant ($p < 0.01$). With regeneration initiated after a 4-week callus induction period, percentage embryogenic calli formation ranged between 45.17% (cv. Hawk) and 91.28% (cv. Sivas-111/33). Twelve genotypes gave a minimum of 60% regeneration potential (Table 1). A value of 60% for regeneration potential was chosen as a minimum since genotypes

Table 1. *In vitro* response of 14 current winter wheat cultivars grown in Turkey in comparison to model genotype (Bobwhite) with regeneration initiated after 4-week callus induction period

Cultivars	Callus formation (%)	Callus fresh weight (mg)	Embryogenic calli (%)	Plantlets per cultured embryo
Bobwhite	99.54 ^{a}	45.571 ^{a}	99.75 ^{a}	15.25 ^{a}
Sivas-111/33	81.76 $^{c-g}$	26.412 $^{c-g}$	91.28 ^{c}	8.66 ^{d}
Kıraç-66	63.57 $^{ı-m}$	21.125 $^{e-ı}$	84.30 ^{fg}	7.39 $^{d-g}$
Haymana 79	69.82 $^{f-j}$	18.333 $^{g-l}$	86.89 ^{de}	6.77 $^{e-h}$
Conkesme	62.46 $^{ı-m}$	21.811 $^{d-ı}$	88.89 ^{d}	6.56 $^{f-ı}$
Sabalan	84.82 ^{bdc}	22.067 $^{d-ı}$	82.60 ^{g}	5.54 $^{g-k}$
Lancer	67.87 $^{g-k}$	15.500 $^{h-l}$	76.77 ^{h}	5.34 $^{g-l}$
Tir	85.23 ^{bcd}	26.783 $^{c-g}$	73.37 ^{kj}	4.88 $^{j-p}$
Aytın-97	70.47 $^{e-j}$	22.778 $^{d-h}$	64.51 ^{m}	4.06 $^{l-t}$
Karasu-90	64.00 $^{h-l}$	25.383 $^{c-g}$	67.24 ^{l}	3.72 $^{m-t}$
Harmankaya	60.49 $^{j-n}$	32.488 ^{cb}	64.04 ^{m}	3.22 $^{n-t}$
Süzen	70.57 $^{e-j}$	11.292 ^{kl}	56.47 ^{n}	2.54 $^{p-t}$
Sürak	45.00 ^{o}	25.296 $^{c-g}$	64.10 ^{m}	2.08 $^{q-t}$
Hawk	84.14 ^{cde}	11.316 ^{kl}	45.17 ^{q}	1.77 ^{rst}
Kırik	62.17 $^{ı-n}$	33.360 ^{cb}	45.80 ^{q}	1.32 st

*The same letters within the same column are not significantly different.

with values less than this are likely to produce low numbers of plants after transformation. Two genotypes; Kırik and Hawk demonstrated poor embryogenic calli formation and had less than 60% regeneration potential. Plant regeneration through somatic embryogenesis was observed from calli of all 14 winter wheat after a 4-week callus induction period. Number of plantles produced per embryo was greatly influenced by genotypes ($p<0.01$) and ranged from 1 to 15. Genotypes with the highest number of regenerated plantles per embryo were Bob White (15.25), Sivas-111/33 (8.66), Kıraç-66 (7.36), Haymana 79 (6.77) and Conkesme (6.56).

There were also significant differences among genotypes based on callus fresh weight ($p < 0.01$). Genotype with the highest callus weight was Bobwhite (45.57 mg). Genotypes having the lowest callus weight were Hawk (11.31 mg) and Süzen 11.29 mg).

Genotypes reduced the number of regenerated plantlets by 8–100%, when the callus induction period was extended from 4 to 8 weeks. Table 2 summarizes the number of plantlets produced per embryo cultured after 4 and 8 weeks callus initiation period and percentage reduction in plantlets regeneration after 8 weeks callus initiation period. Genotypes which produced more than 1 plantlet per cultured of embryo after an extended (8 weeks) callus initiation period were four winter wheats; Sivas-111/33 (5.42), Kıraç-66 (4.80), Haymana 79 (1.50), Conkesme (1.20) and one spring wheat (model genotype) Bobwhite (13.20 plantlets per cultured embryo). These four winter wheat genotypes have been selected for initial *Agrobacterim*-mediated transformation experiments due to their high regeneration potentials. Five genotypes; Sabalan, Lancer, Tir, Aytın-97, and Karasu-90 were produced less than 1 plantlets per cultured embryo after an extended (8 weeks) callus induction period, while five genotypes; Harmankaya, Süzen, Sürak, Hawk, and Kırik completely lost their regeneration potential and they did not produce any plantlets when the callus induction period was extended from 4 to 8 weeks.

Table 2. Comparison of 4-week and 8 week callus induction period (CIP) based on regenerated plantlets per cultured embryo

Genotypes	Plantlets per cultured embryo (4 week CIP)	Plantlets per cultured embryo (8 week CIP)	Reduction in plantlets production (%)
Bob White	15.25	13.20	13.44
Sivas-111/33	8.66	5.42	37.41
Kirac-66	7.39	4.80	35.05
Haymana 79	6.77	1.50	77.84
Conkesme	6.56	1.20	81.71

DISCUSSION

In order to efficiently complement traditional wheat breeding with genetic transformation technology it will be desirable to introduce transgenes into the ideal genetic background. The number of wheat genotypes that can be stably transformed is limiting this approach mainly due to poor tissue culture performance (Takumi and Shimada, 1997). Variation for tissue culture performance in wheat is largely genetically controlled (Shimada 1978, Sears and Deckard 1982, Carman et al. 1988, Redway et al. 1990, Fennell et al. 1996, Machii et al. 1998, Viertel et al. 1998, Barro et al. 1999, Aydin et al. 2011). Consequently, our objective was to identify current winter wheats grown in Turkey with excellent tissue culture performance.

Fourteen current winter wheat cultivars grown in Turkey (Tables 1 and 2) were evaluated for tissue culture response from immature embryo explants. Regenerable callus cultures could be initiated from all genotypes analyzed. The callus induction frequency of cultured embryos ranged between 45% (cv. Sürak) and 99.54% (cv. Bobwhite). Somatic embryogenesis of induced calli ranged between 45.17% (cv. Hawk) and 99.75% (cv. Bobwhite). Significant genotypic differences were observed for callus induction and somatic embryogenesis. Statistical analysis revealed that the best differentiation parameter between genotypes in our experiments was the number of regenerated plantlets per cultured explant. Viertel et al. (1998) also found this parameter as most conclusive for the comparison of regeneration potential in spring wheat.

It is known that *in vitro* responses are genotype dependent (Maheshwari at al. 1995, Ahloowalia et al. 1982, Carman et al.1988, Haliloglu et al. 2005) and our results agreed with this finding. Maddock et al. (1987) found that embryo formation and shoot regeneration varied from 12% to 96% in 25 cultivars. He et al. (1990) reported that production of plants

from embryogenic and nonembryogenic calli were genotype dependent.

On the same medium, some genotypes formed embryogenic calli and regenerated plantlets while others produced less embryogenic calli and were incapable of regeneration. This variation in genotypic response on the same medium indicates that there are genetic components controlling traits related to callus formation and plantlets regeneration as have been suggested by others (Galiba et al. 1986, Mathias and Fukui 1986, Kaleikau et al. 1989). Studies on ND7532 using monosomic analysis identified homoelogous group 2 chromosomes, in particular chromosome 2D, contained genetic factors promoting regenerable callus formation and callus growth rate (Kaleikau et al. 1989). Analysis of immature embryo cultures derived from 4B chromosome substitution lines has indicated that homoeologous group 4 chromosomes, specifically chromosome 4B, influence regenerative capacity (Mathias and Fukui 1986). Kaleikau et al. (1989) assessed the significant differences between the aneuploid lines of group 2 chromosomes and the euploid control for the expression of both regenerable callus formation and callus growth rate. They further speculated that the location of a major regulatory gene controlling the expression of tissue culture response genes might be on chromosome 2BL. Mathias and Atkinson (1988) suggested that allelic variation in wheat at the *Rth/Gai* gene (reduced height/gibberellic acid insensitivity) may effect callus growth, somatic embryogenesis and plant regeneration via an effect on hormonal metabolism.

The majority of genotypes reduced the number of regenerated plants significantly when the callus induction period was extended from 4 to 8 weeks (Tables 2). A decline in regeneration potential of wheat with extended callus culture periods was also described by Fennell et al. (1996) and might be at least partially due to the mutagenic effect of the auxins (Deambrogio and Dale 1980, Murata 1989). Therefore, a short callus culture period seems to be important for the successful transformation of a wide range of current wheats.

The purpose of this study was to identify wheat genotypes with the highest potential for regeneration and select the best lines for *Agrobacterium*- mediated transformation experiments. Four genotypes, 'Sivas-111/33, Kirac-66, Haymana 79, and Conkesme', have been selected for initial *Agrobacterim*-mediated transformation experiments due to their high regeneration potentials.

Explant genotypes still restrict the application of *Agrobacterium*-mediated transformation due to its host range specificity. Boulton et al. (1989) found that agroinfection of maize was essentially genotype dependent. Similarly, spring wheat was favored by Mooney et al. (1991). Therefore, this study takes the first step towards finding the better genotypes that have good response to *in-vitro* culture and will serve the better genotypes to be used in transformation studies to overcome the *Agrobacterium* host specificity barrier.

Yield lag is a concern in production of transgenic crops and results when new transgenic cultivars yield less than newer elite cultivars. Yield lag is due to the time required for backcrossing transgenes into elite cultivars. In wheat transformation, there are only few cultivars that have been extensively used for transformation due to their good response to tissue culture (e.g. Bobwhite). Therefore, quite long time is required to transfer the transgenes into elite winter lines by means of classical breeding methods such as backcrossing. By the time the transgenic cultivar is released, there are new cultivars, which have higher yield.

In this study, we focused on screening the cultivars used in our breeding program to serve as an explant in our transformation efforts in order to overcome the yield lag problem of transgenics and gain time by reducing the backcross procedure. In this research, we identified winter wheat cultivars that may be suitable for transformation.

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