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Research Article

Callus Production and Plant Regeneration from Anther Culture of Some Turkish Barley Cultivars

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Abstract: Anther culture of four different barley cultivars (*Hordeum vulgare* L. cvs. Anadolu, Cumhuriyet-50, Obruk-86 and Tokak-157/37) currently cultivated in Turkey has been investigated. Two different culture media (BAC3 and FHG) were used and the effect of a 21-day cold pretreatment was studied. Androgenesis on BAC3 medium was evaluated statistically in all genotypes and the percentage of anther response and the frequency of calli production were found significantly different ($P<0.01$) between the pretreatments in all cultivars. The cultivar Cumhuriyet-50 displayed the highest number of transferable calli without cold pretreatment (70.1%) and 51.0% of the regenerated plants formed roots. For plants without roots, high auxin level (NAA and IAA) was used to stimulate root formation.

Key Words: Anther culture, *Hordeum vulgare* L., androgenesis, plant regeneration

Bazı Türk Arpa Çeşitlerinin Anter Kültüründen Kallus Üretimi ve Bitki Regenerasyonu

Özet: Anter kültürü tekniğinin ülkemizde tarımı yapılan dört arpa çeşidine (*Hordeum vulgare* L. cvs. Anadolu, Cumhuriyet-50, Obruk-86 and Tokak-157/37) uygunluğu araştırılmıştır. İki farklı kültür ortamı (BAC3 ve FHG) kullanılmış ve 21 gün soğuk önişlemin etkisi araştırılmıştır. Tüm çeşitlerin androgeneze uygunluğu BAC3 kültür ortamında istatistiksel olarak değerlendirilmiş olup önişlem uygulamalarında anter tepkisinin oranı ve üretilen kallus frekansı arasındaki farklılık önemli ($P<0.01$) bulunmuştur. Cumhuriyet-50, soğuk önişlem uygulanmadığı koşulda en yüksek transfer edilebilir kallus (70.1%) ve kök oluşturabilmiş bitki 50.1% üretmiştir. Kök formasyonunu oluşturmayan bitkilerde, kök oluşumunu teşvik için yüksek düzeyde oksin (NAA ve/veya IAA) kullanılmıştır.

Anahtar Sözcükler: anter kültürü, *Hordeum vulgare* L., androgenez, bitki rejenerasyonu

Introduction

In the last decades anther culture, the induction of callus from cultured anthers and the production of haploid plants from calli, has been developed by many researchers (1-3). By doubling chromosomes in haploid plants it is possible to obtain completely homozygous lines in a short time thereby providing a method for speeding up and increasing the selection efficiency (4). The most important factor appears to be the genotype response to androgenesis in the development of callus from microspores. It appears that optimum media and pretreatment conditions vary from genotype to genotype. Two main approaches have been used in an attempt to improve anther culture response of barley genotypes. The first approach has been to select responsive genotypes and use them in barley breeding programs. The second has been to identify physiological and environmental factors which may influence the response of anthers to culture (3).

In this study, four Turkish barley (*Hordeum vulgare* L.) cultivars Anadolu, Cumhuriyet-50, Obruk-86, Tokak-157/37 were used for anther culture in order to evaluate their capacity for callus production and plant regeneration by using two different media as well as a 21-day cold pretreatment.

Materials and Methods

H. vulgare L. (2n=14) cvs. Anadolu, Cumhuriyet-50, Obruk-86, Tokak-157/37 were used as plant material in this study. All cultivars have two-row spikes and alternative characters. Tokak-157/37 is one of the oldest barley cultivar of Turkey and has been still cultivated in large areas of the middle Anatolia because of its high yield and good quality malt component.

Plants were grown in a glasshouse and irrigated with tap water supplemented with major and minor nutrients. Light was supplied by lamps (10-20klux) on a photoperiod of 16h/8h day/night with a temparature of 12±3°C. The spikes for anther culture were collected when the microspores were at mid- or late-uninucleate stage. Inter-ligule length of the tip of the tiller was used as an approximate indicator of this stage. For cold pretreatment each selected spike was surface sterilized with 70% etanol for 10 min and were kept in a refrigerator at 4°C for 21 days. Spikes were placed in 9 cm petri dishes containing several drops of sterile distilled water to maintain humidity and sealed with parafilm then wrapped with aluminium foil (5). After 21 days, the spikes were selected according to developmental stage of microspores (in a drop of 2% aceto-carmine solution, under the light microscope) and anthers were plated onto two different media.

Preparation of media: The anthers were plated onto a liquid BAC3 medium (6) containing 2 mg/l NAA and 1 mg/l BAP or liquid FHG medium (7) containing 1 mg/l BAP, both being referred to as induction medium. The media components were sterilized with a cellulase membrane filter (pore size is 0.22 μm) and added to autoclaved Ficoll 400 medium. pH of the media was adjusted using 1N NaOH and 1N HCl to 6.2 and 5.8 for induction and regeneration media, respectively. The regeneration medium was agar-solidified with 1 mg/l IAA and 0.5 mg/l Kinetin for BAC3. For FHG, the regeneration medium was not prepared because of lacking of the calli for regeneration.

Usually, anthers of two spikes were used per replication using a completely randomised design. Each replication contained 3 ml of liquid induction medium in a 30 mm diameter petri dishes. Plating density was around 10-20 anthers per ml of medium. The petri dishes were sealed with parafilm and wrapped with aluminium foil. For incubation, plated anthers were placed in an incubator at 27±1°C in dark. Media were replenished by adding 1 ml fresh media after 2 and 4 weeks of incubation.

After 30 to 40d, calli were transferred onto regeneration medium in a 9 cm diameter petri dish. Sixteen-20 embryoids/calli were put in each petri dish and cultured under cool white fluorescent lamps at 22°C with a 16h photoperiod. The number of green and albino plantlets was counted. Then green plantlets were transferred to small pots with a mixture of peat and perlite at a ratio of 3:1 in a growth chamber and then to the greenhouse.

For root development, BAC3 regeneration medium was modified. All inorganic and organic salts were decreased to half of the original components and supplemented with 4 g/l charcoal to promote rooting. Auxin level was increased ten times (10 mg/l NAA or IAA) and 0.5 mg/l Kinetin.

Analysis of variance was performed on all data and comparison of cultivars, media and cold pretreatment were made using Duncan's Multiple Range Test. Experiments were repeated 6-8 times.

Results

The percentage of responsive anthers were found to be significantly different between cultivars on BAC3 medium ($P<0.01$) either with or without cold pretreatment (Table 1). Tokak 157/37 and Cumhuriyet-50 cultivars displayed anther responses of 59.2% and 56.6% respectively without cold pretreatment (Table 2) (Figure 1). After a 21-day cold pretreatment, Tokak and Cumhuriyet anthers were responded 97.6% and 75.3% by producing calli in the BAC3 medium (Table 2). Anadolu and Obruk-86 cultivars responded at 28.2% and 26.4% respectively, without cold pretreatment and 40.4% and 34.5%, respectively, after cold pretreatment (Table 2). The interaction was found to be significant ($P<0.01$) between the percentage of responsive anthers and pretreatment conditions in BAC3 (Table 1).

Calli frequency per 100 plated anthers onto the BAC3 medium were found in each cultivar with and without cold pretreatment (Table 2). There were significant differences between cultivars ($P<0.01$) and between pretreatment conditions ($P<0.01$) (Table 1). The Tokak 157/37 genotype produced highest frequency of calli either without cold pretreatment (172.8) or after a 21 days cold pretreatment (571.6) (Table 2) (Figure 2). The interaction between calli production of genotypes and pretreatment conditions was not significant (Table 1).

Plant regeneration was observed in BAC3 medium in all cultivars (Table 2). All cultivars produced plants after

Table 1. Aralysis of variance for BAC3 medium including responsive anthers and callus production in four different cultivars

Variation Sources	d.f.	Anthers response	d.f.	Callus production
Cultivars	3	0.000**	3	0.000**
Pretreatments	1	0.000**	1	0.000**
PxC	3x1	0.002**	3x1	$0.066^{n.s.}$

** significant at P<0.01

n.s. non significant

cold pretreatment and the highest green plant frequency was found in Cumhuriyet-50 (8.9) (Figure 3). Anadolu and Obruk-86 produced 0.4% and 1.2% green plants after cold pretreatment respectively (Table 2). However, Anadolu and Obruk-86 did not produce plants without cold pretreatment.

Tokak 157/37 and Cumhuriyet-50 produced 1350 and 1332 calli after cold pretreatment and 584 and 579 calli without cold pretreatment, respectively (Table 3). In Cumhuriyet-50 cultivar, the highest frequency of calli

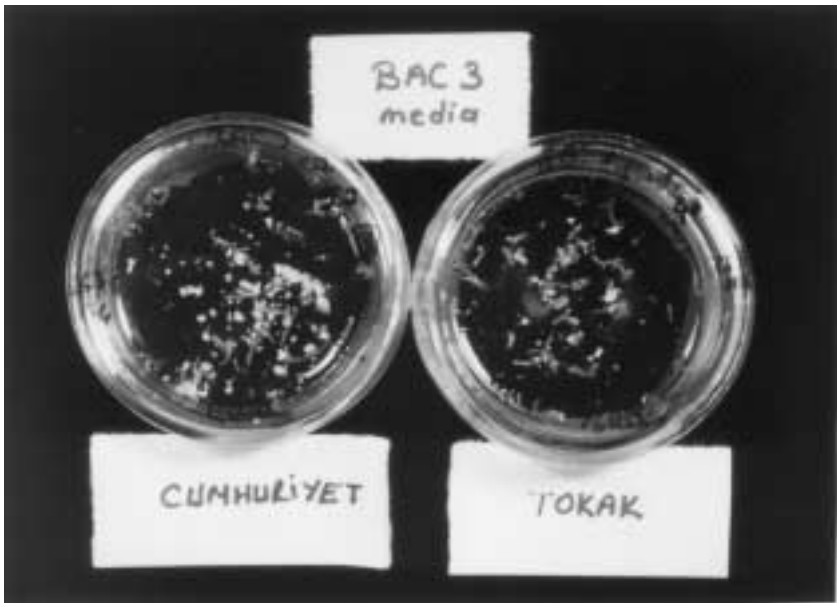

Figure 1. Callus production on liquid BAC3 medium in Tokak-157/37 and Cumhuriyet-50

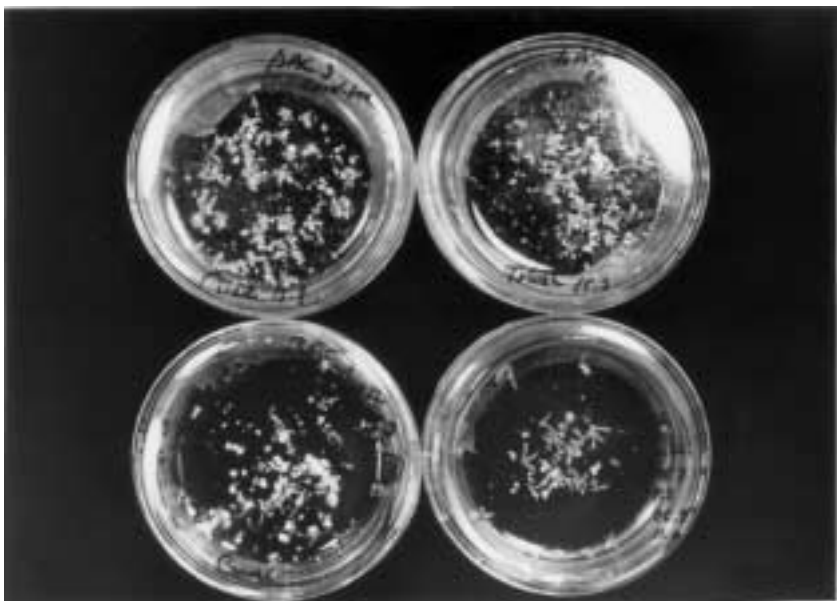

Figure 2. Callus production on BAC3 medium without (0) and after (21 days) cold pretreatment in Tokak - 157/37 and Cumhuriyet - 50 cultivars. In the left, Cumhuriyet-50, after (21 days) cold pretreatment (above), without (0 day) cold pretreatment (below). In the right, Tokak 157/37, after (21 days) cold pretreatment (above), without (0 day) cold pretreatment (below).

Table 2. The response of Turkish barley cultivars to androgenesis without (Oday) and with cold pretreatment (21 days) in two different media

Without cold pretreatment (0 day)							
Cultivars	Medium	Number of anthers plated	Anther* response (%)	Calli** production (%)	Plant green (%)	regeneration** albino (%)	total (%)
Tokak-157/37	BAC3	338	59.2[a]****	172.8[a]	4.1	5.6	9.7
	FHG	216	0.9	3.2	-***	-	-
Cumhuriyet-50	BAC3	362	56.6[a]	160.0[a]	6.4	22.7	29.1
	FHG	410	2.5	3.7	-	-	-
Anadolu	BAC3	117	28.2[ab]	24.0[b]	-	-	-
	FHG	114	1.4	1.4	-	-	-
Obruk-86	BAC3	169	26.4[b]	36.0[b]	-	-	-
	FHG	174	5.9	8.9	-	-	-
With cold pretreatment (21 days)							
Tokak-157/37	BAC3	254	97.6[a]	571.6[a]	3.9	9.5	13.4
	FHG	366	6.3	13.7	-	-	-
Cumhuriyet-50	BAC3	404	75.3[b]	329.7[b]	8.9	22.8	31.7
	FHG	405	8.1	18.0	-	-	-
Anadolu	BAC3	230	40.4[c]	103.3[c]	0.4	1.3	1.7
	FHG	155	5.8	7.4	-	-	-
Obruk-86	BAC3	165	34.5[c]	94.0[d]	1.2	4.2	5.4
	FHG	119	6.7	3.4	-	-	-

* The percentage of responsive anthers

** The frequency of calli and regenerated plants/100 plated anthers

*** No observation

**** Values with the same letters are not significantly different at P=0.01 according to Duncan's Multiple Range Test in each pretreatment separately.

were transferred (70.1) without cold pretreatment. Also, the Tokak 157/37 variety produced the highest green plant regeneration frequency (9.3) without cold pretreatment, according to the transferred calli frequency (Table 3).

Tokak 157/37 cultivar produced 17 green and 43 albino plantlets and 12 plants (70.6%) of 17 produced roots in BAC3 regeneration medium (Figure 4). In Cumhuriyet-50, 25 plants (51.0%) of 49 green regenerants produced roots, 8 plants survived in the greenhouse and 71 seeds reached maturity. The plantlets of Tokak-157/37, Anadolu and Obruk-86 did not survived to the greenhouse (Table 4).

Discussion

Response to androgenesis for a number of crops including barley is known to be strongly genotype dependant and influenced by numerous environmental factors. In barley, Foroughi-Wehr et al (1) reported that the variety Dissa was more amenable to androgenesis than nineteen other genotypes. Luckett and Smithard (8)

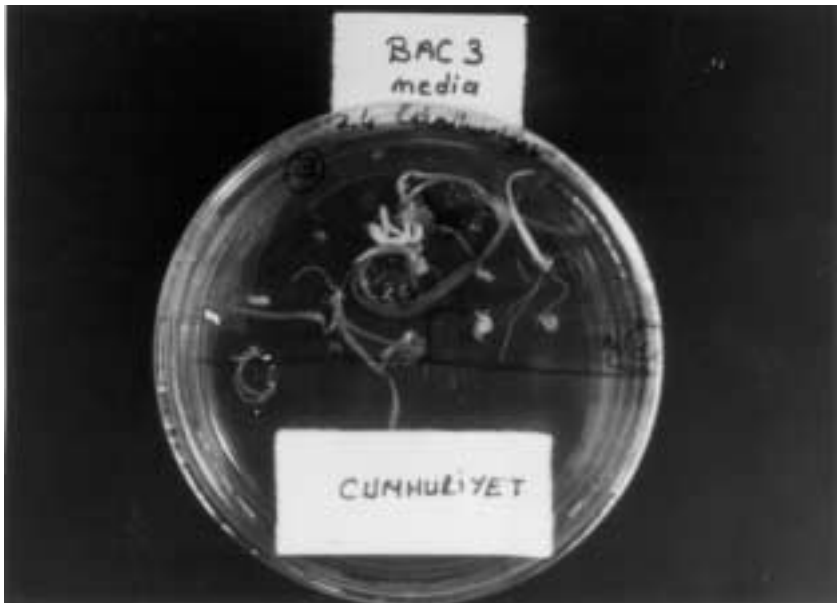

Figure 3. Plant regeneration (green and albino) in Cumhuriyet-50 cultivar.

Table 3. Calli transferred onto BAC3 regeneration medium for plant regeneration

Cultivars	Cold pretreat. (days)	Number of calli produced	Number of calli transferred	Calli* transferred (%)	Plant regeneration** green (%)	albino (%)	total (%)
Tokak-157/37	0	584	151	25.8	9.3	20.2	29.5
	21	1350	306	22.6	3.3	19.7	23.0
Cumhuriyet-50	0	579	406	70.1	5.7	20.2	25.9
	21	1332	467	35.0	7.7	19.7	27.4
Anadolu	0	28	-	-	-***	-	-
	21	237	32	13.0	3.1	9.4	15.5
Obruk-86	0	64	14	21.8	-	-	-
	21	155	39	25.1	5.1	17.9	23.0

* The percentage of calli transferred onto regeneration medium

** The frequency of plantlet/100 transferred calli

*** No observation

Table 4. Number of plants regenerated and root formation in green plants.

Cultivars	Number of plant regenerated			Number of green plants		
	Green	Albino	Total	Rooted	(%)	Non-rooted
Tokak-157/37	17	43	60	12	70.6	5
Cumhuriyet-50	49	174	223	25	51.0	24
Anadolu	1	3	4	-	-	1
Obruk-86	1	7	8	-	-	1

Figure 4. Plant regeneration in petri dishes and root formation in green plantlets in tubes.

have also reported differences in green plant production from anther culture of Australian barley genotypes. Knudsen et al (9) demonstrated that Igri, a winter type genotype, gave the highest number of green plantlets. Kintzios and Fischbeck (10) have observed that winter barley cultivars and F2 and/or backcross progeny of some lines produced an average of 4 green regenerants per 100 anthers plated in their study. In our study, Cumhuriyet-50 produced the highest number of green plantlets although Tokak-157/37 was the most responsive cultivar with respect to calli production (Table 2).

Both of media were prepared by using their original components (6, 7) and BAC3 medium has given more succesful results than FHG medium to androgenesis for four Turkish cultivars (Table 2). Cai et al (11) have studied various combinations of growth regulators for BAC3-Ficoll containing medium. They have showed that the combination of the auxin NAA (2 mg/l) and the cytokinin BAP (1 mg/l) resulted in embryoid formation much greater than media containing 2,4-D combined either with the cytokinin zeatin riboside or BAP. In the present study, 2 mg/l NAA and 1 mg/l BAP were used as growth regulators in BAC3 induction medium and 1 mg/l BAP and no auxin was used as growth regulators in FHG induction medium (7). In FHG, the highest calli production were found in Cumhuriyet-50 (18.0%) and in Tokak 157/37 (13.7%) after cold pretreatment (Table 2).

In barley anther culture, preparation of liquid induction medium has been achieved by using Ficoll (2). Liquid medium provides aeration to calli on the surface without sinking in the bottom. It is a tremendous possibility to float culture of anthers on the liquid medium and to produce calli on the medium surface (2, 11). It can be regarded as a reason why Tokak-157/37 cultivar has given 172.8 calli without cold pretreatment and 571.6 calli after 21 days cold pretreatment plated per 100 anthers onto the medium.

Temparature-stress pretreatments has been used to enhance the production of plants from cultured anthers in barley as well as other crop plants (5, 14, 15). To increase the effectiveness of the four Turkish varieties, 21-day cold pretreatment was used before anthers were plated onto induction media (Figure 2). Huang and Sunderland (5) reported the effectiveness of different cold pretreatments and demonstrated that H. vulgare L. cv. Sabarlis produced more callus after 28 days at 4˚C. Powell (15) reported that also 21 days pretreatment at 4˚C could be optimal for the induction of anther response in different spring barley cultivars.

The highest number of calli transferred in Cumhuriyet-50 cultivar onto the BAC3 regeneration medium either without cold pretreatment or after 21 days cold pretreatment (Table 3). Cumhuriyet-50 produced 579 calli without cold pretreatment and 70.1 percent of calli (406) were transferred to regeneration medium for differentiation (Table 3). Marsolais and Kasha (16) described the two different calli structure in anther culture, W-type calli (calli with predominantly loose, watery, transparent cells) and E-type calli (sometimes referred to as embryoids, with compact, dense cells, white to light yellow in colour, and ranging in shape from globular to multilobed). In this study, calli similar to E-type were used for regeneration.

The suitable composition and concentration of exogenous hormones would affect the developmental pathway of pollen, callus induction frequency and morphogenesis (17). Nevertheless, the relative ratio of IAA, NAA and Kinetin also played a role in differentiation. In our study, to provide strong root formation in the green regenerant plants, high levels of auxin (NAA or IAA) in combination with Kinetin were used in the regeneration medium (Table 4). Other workers have also observed that IAA favoured root elongation and also that NAA benefited the sturdy growth of root (17).

The elapsed time from anther plating to callus appearance varied between 25 and 60 days and 93% of the calli appeared in the first 30 days of culture in this study. Franzone et al (18) have reported that various barley varieties produced 85% of calli in the first 70 days of culture. The earliest green plant regeneration occurred within 7 and 14 days in Cumhuryet-50 with and without cold pretreatment, respectively.

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