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REGENERATION CAPACITY OF MATURE EMBRYO-DERIVED CALLUS IN BARLEY (*HORDEUM VULGARE* L.)

FILIZ GÜREL,* ÖZGE KARAKAŞ, GÜLRUH ALBAYRAK and ŞULE ARI

Department of Molecular Biology and Genetics, Istanbul University,
Vezneciler, 34134 Istanbul, Turkey

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In this study, induction of regenerable callus from mature embryos in eight Turkish barley varieties was analysed by using different plant growth regulators (PGRs). Varying concentrations (0.5–4 mg l^{-1}) of 2,4-dichlorophenoxyacetic acid (2,4-D) and dicamba (3,6-dichloro-*o*-anisic acid) were tested for callus induction from mature embryos. Highest percent of callus induction was observed in Bornova 92 variety (98.3%) on MS medium supplemented with 4 mg l^{-1} dicamba. Calli were transferred to regeneration media with 0.5 mg l^{-1} dicamba, 0.5 mg l^{-1} zeatin riboside (ZR) and 2 mg l^{-1} thidiazuron (TDZ). Low concentrations of dicamba induced multiple shoots during callus regeneration. When the effect of precultivation with 2,4-D or dicamba on the shoot induction were evaluated, lower concentrations (< 4 mg l^{-1}) of auxins have been found optimal. On the regeneration medium with 0.5 mg l^{-1} dicamba, shoots were able to elongate up to 20 cm and shoot numbers were between 1–23 per callus. The use of ZR led to formation of short shoot buds and somatic embryos in 2 weeks period. The effect of TDZ was different from other PGRs by inducing green solid sectors on calli surfaces (Total 51 sectors/20 callus/Akhisar variety). Five plantlets have been grown from these solid cell clumps and transferred to specific media for root formation. As a result, five varieties (Süleyman Bey, Bornova 92, Vamık Hoca, Kaya and Akhisar) tested in our study showed the potential to produce regenerable callus by using low amounts of dicamba or TDZ. The optimization process starts from culturing embryos to plantlet formation took nearly 4 weeks.

Keywords: Barley – callus – mature embryo – dicamba – TDZ

INTRODUCTION

Barley (*Hordeum vulgare* L.) is used mainly in animal feeding and brewing industries in the world. Its use in human food industry is increased recently because of the nutritional benefits of barley grains.

* Corresponding author; e-mail: filiz@istanbul.edu.tr

Barley is the second crop in Turkey after wheat with the annual production of 9.5 million tons with 2653 kg/ha yields according to FAO statistics (2006). Improvement of Turkish barley varieties for yield and grain qualities is in progress by conventional methods. Water shortage, salt-stress and pathogens (*Fusarium culmorum*, *Ustilago hordei*, etc.) are the major limitations for barley breeding in Turkey. In order to overcome these problems, biotechnological work has also been carried out by transformation [13], genotyping [3, 4, 12] and functional genomics [19] studies. However, there are no reports concerning the tissue culture potentials of native barley genotypes which can be defined for molecular applications.

Barley callus is a potential target for gene transfer although it is not commonly used due to its low regeneration efficiency. Tested explants for regenerable callus were apical meristems [9, 22], immature embryos [6, 7] and mature embryos [11, 15, 21]. Among them, mature embryos are attractive as an explant source because of their long time availability. They can be isolated abundantly from mature seeds and used extensively to study tissue culture conditions. Intact embryos also have high germination frequency and were used in direct gene transfers such as electroporation [13, 14]. Plant regeneration from mature embryos by a callus phase was barely succeeded in embryogenic [11] or non-embryogenic cultures [21]. In the former study, somatic embryos were seen on calli after 22 days and transferred to regeneration medium with low auxin concentrations in order to obtain plantlets. However, plantlet regeneration was emerged in a low frequency (33/173) after 45 days. This regeneration process is relatively difficult and needs long culture periods that results in somaclonal variations [11]. Therefore, the duration between culture initiation and shoot induction should be shortened to avoid genomic instabilities in callus phase.

The regeneration capacity in barley is highly affected by genotypes [16, 21], medium compositions [6] and karyotype reconstruction [1]. First, the genotypes with higher regeneration capacity can be selected and then the efficiency can be improved by media ingredients. Comparative studies demonstrate that the response of barley genotypes to media is more specific than for wheat and triticale [20]. Therefore, selected genotypes (such as Golden Promise) and economically important varieties (Morex, Clipper, CDC Dawn, etc.) were evaluated in respect of tissue culture response [2, 6, 10, 21]. Apparently, both the auxin type and its concentration deter-

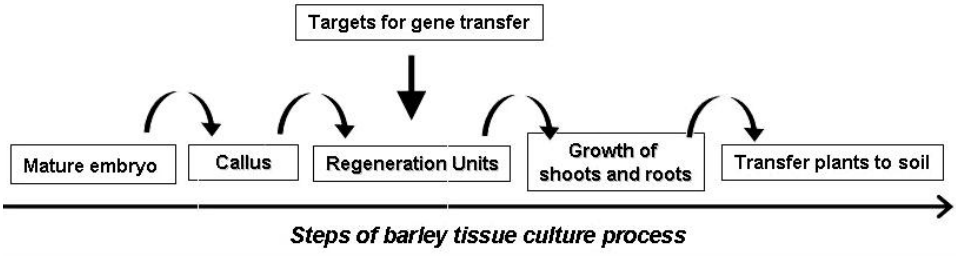

Fig. 1. Experimental steps for obtaining barley plants from mature embryos

mine different patterns of cell proliferation and morphogenesis on callus. For example, Akula et al. [2] tested diverse plant growth regulators for initial callus induction and regeneration in Australian malting barley genotypes. The unique response of each variety to the media combinations was clearly underlined in that study.

The aim of our study was to examine the effects of several growth regulators over callus regeneration in selected Turkish genotypes. Different plant growth regulators can be applied during the tissue culture process which covers mainly four experimental steps (Fig. 1). We tested 2,4-D and dicamba for the first step (callus induction from mature embryos) and dicamba, TDZ and ZR for the second step (regeneration from callus). A shortened regeneration process was also taken into consideration in order to avoid genomic instabilities in barley cells.

MATERIALS AND METHODS

Seed material

Dry seeds of barley varieties Kaya, Vamık Hoca, Şerife Hanım, Bornova 92, Zafer 160, Akhisar, Süleyman Bey and Tokak were kindly supplied from Aegean Agricultural Research Institute (Menemen), İzmir (Turkey). Bornova 92, Kaya, Süleyman Bey and Şerife Hanım are two-rowed and the rest of the varieties are six-rowed.

Tissue culture

Mature embryos were excised from seeds after surface sterilization as described previously [11]. Shoot and root apex segments of mature embryos were injured by a scalpel blade to prevent germination. Basal salts of Murashige and Skoog (MS) [17] were supplemented with 3% (w/v) sucrose, 1 ml of MS vitamin mixture and 0.8% agar with a pH of 5.75. Each Petri dish (11.5 cm in size) contained 10 embryos with scutella contacting to medium. 2,4-D (Sigma D-4517) and dicamba (Sigma/Riedel-de Haen 45430) were sterilized by using filter and added to autoclaved medium at the concentrations of 0.5, 1.5, 2 and 4 mg l^{-1}. Numbers of primary calli were counted after 10 days in each variety. Calli less than 0.5 cm in size were excluded. Fresh weights of 10 calli from each variety were determined at the end of ten days. Selected primary calli on 2 and 4 mg l^{-1} concentrations of dicamba and 2,4-D media were transferred to the following media: MS + 0.5 mg l^{-1} dicamba, MS + 0.5 mg l^{-1} ZR and MS + 2 mg l^{-1} TDZ. All kind of cultures were kept in a growth chamber (Heraeaus, Vötsch, No: 440/0026/86) at 25 ± °C under a photoperiod of 8/16 (dark/light), 1400 lux. Shoots regenerated on calli were counted 2 weeks after transfer to each medium.

Statistical analyses

Each Petri dish included 10 embryos and a total of 30 embryos were evaluated from each variety to determine callus induction. Experiments were separately repeated in 3 times. Analysis of variance (ANOVA) was performed for 2 kinds of media (MS + dicamba and MS + 2,4-D) by using *Statistica* computer program. One-way ANOVA results show the relation between callus induction percentage and plant growth regulator concentrations.

RESULTS

Callus induction

Callus induction from mature embryos was observed in all varieties from 5 to 7 days after culture initiation. Vigorously growing callus emerged from mature embryos or in some cases both callus induction and germination took place on the same embryo. In that case, callus part was dissected and transferred to fresh media. After 10 days, the weight of fresh callus increased in different amounts while the degree of increase was higher in dicamba than 2,4-D. For example, calli formed in case of Tokak cultivar weighed approximately 82.25 mg on 2,4-D and 170 mg on dicamba supplemented media.

Callus induction frequency for each variety is given in Table 1. Embryo numbers that induce callus gradually increased in parallel with dicamba concentrations (Table 1a). Bornova 92 and Tokak were the most responsive genotypes by giving highest ratios of callus induction, 98.33% and 93.33, respectively. The use of 2,4-D was less effective in callus induction than dicamba at all concentrations (Table 1b) and callus induction was hardly exceeding 50% in Bornova 92 and Tokak. There was positive correlation between the auxin concentrations and callus induction frequency in terms of F values (Table 1). On the basis of one-way Anova analysis, the increase in callus numbers by auxin concentration was significant in most of the genotypes at the $p < 0.001$ level.

Callus morphology seemed to be homogeneous in all varieties at the beginning of culture period. After 2 weeks, different callus could be observed in the varieties treated by dicamba. For example, Süleyman Bey and Tokak varieties tend to produce type B callus referred as compact and nodular [6]. Both type A and type B callus were seen in remaining varieties, only in the Akhisar variety were most of the calli soft and translucent (type A).

Shoot regeneration

The use of ZR, dicamba and TDZ were tested for their effects on the efficiency of shoot regeneration. Use of 0.5 mg l^{-1} dicamba led to formation of white compact cal-

Table 1
Callus induction in Turkish barley varieties

(a)

Variety	% of callus induction ±SE					
	MS + Dicamba (mg l^{-1})				One-way ANOVA	
	0.5	1.5	2	4	*F* value	p
Süleyman Bey	48.33 ± 2.04	63.33 ± 2.04	70 ± 3.54	73.33 ± 4.09	19.666	0.000476
Bornova 92	61.66 ± 2.04	90 ± 3.54	93.33 ± 2.04	98.33 ± 2.04	65.111	0.000006
Akhisar	46.66 ± 4.09	48.33 ± 2.04	61.66 ± 2.04	70 ± 3.54	19.851	0.000460
Vamık Hoca	25 ± 3.54	55 ± 3.54	66.66 ± 2.04	75 ± 3.54	68.9	0.000005
Zafer 160	40 ± 3.54	60 ± 3.54	63.33 ± 2.04	70 ± 3.54	24	0.000236
Şerife Hanım	6.66 ± 2.04	50 ± 3.54	58.33 ± 2.04	75 ± 3.54	199.777	0
Tokak	58.33 ± 2.04	78.33 ± 2.04	88.33 ± 2.04	93.33 ± 2.04	86.25	0.000002
Kaya	45 ± 3.54	75 ± 3.54	81.66 ± 2.04	83.33 ± 2.04	57.458	0.000009

(b)

Variety	% of callus induction ±SE					
	MS + 2,4-D (mg l^{-1})				One-way ANOVA	
	0.5	1.5	2	4	*F* value	p
Süleyman Bey	11.66 ± 2.04	21.66 ± 2.04	26.66 ± 2.04	41.66 ± 2.04	56.25	0.000010
Bornova 92	13.33 ± 2.04	26.66 ± 2.04	41.66 ± 2.04	55 ± 3.54	67	0.000005
Akhisar	1.66 ± 2.04	18.33 ± 2.04	21.66 ± 2.04	33.33 ± 2.04	61.583	0.000007
Vamık Hoca	3.33 ± 2.04	16.66 ± 2.04	26.66 ± 2.04	30 ± 3.54	34.444	0.000064
Zafer 160	5 ± 0	11.66 ± 2.04	18.33 ± 2.04	30 ± 3.54	32.733	0.000077
Şerife Hanım	8.33 ± 2.04	21.66 ± 2.04	31.66 ± 2.04	36.66 ± 2.04	56.25	0.000010
Tokak	8.33 ± 2.04	31.66 ± 2.04	38.33 ± 2.04	63.33 ± 2.04	184.25	0
Kaya	3.33 ± 2.04	21.66 ± 2.04	23.33 ± 2.04	46.66 ± 5.41	45.433	0.000023

Percent callus induction in eight barley varieties. Two kinds of media combinations (MS + dicamba, MS + 2,4-D) were given in (a) and (b). One-way ANOVA results (*F* values) show the relation between callus induction and auxin concentrations.

Table 2
Shoot induction on calli

Precultivation	No. of callus with shoots/calli (0.5 mg l^{-1} dicamba)			
	2,4-D		Dicamba	
	2 mg l^{-1}	4 mg l^{-1}	2 mg l^{-1}	4 mg l^{-1}
Variety				
Süleyman Bey	15/17	12/18	19/19	12/20
Bornova 92	16/20	17/19	19/20	9/20
Akhisar	15/20[a]	14/20	6/20	–/14
Vamık Hoca	15/19	10/19	14/20	1/20[b]
Zafer 160	15/20[a]	11/20[b]	18/20	–/20
Şerife Hanım	10/20	3/19	11/20	9/19[b]
Tokak	13/19	8/20[b]	17/20	3/20
Kaya	12/18	9/14	4/18	4/20

Effects of 2,4-D and dicamba precultivation for shoot induction on calli transferred to MS + 0.5 mg/L dicamba regeneration medium. Shoots were counted on calli after 2 weeks. [a] Shoot length: 16–20 cm; [b] Shoot length: 1–2 cm.

lus with multiple shoots. Particularly, Süleyman Bey and Bornova 92 varieties were responsive to produce calli with shoot initiations (Fig. 2A) which rapidly elongated in 9–10 days (Fig. 2B). Precultivation with 2,4-D or dicamba before transferring to regeneration media had affected shoot induction efficiency in the following days. Table 2 shows the numbers of calli with shoots developed on 0.5 mg l^{-1} dicamba media after 2 weeks. Numbers of calli with shoots were slightly higher in 2 mg l^{-1} auxin concentration than 4 mg l^{-1}. Four mg l^{-1} dicamba precultivation decreased the shoot induction in subsequent culture period. Maximum shoot numbers were seen in Bornova 92 [1–23]. Shoot lengths were between 1 and 20 cm at 0.5 mg l^{-1} dicamba treatment.

On MS with ZR medium, regenerable callus was observed in most of the varieties specifically in Vamık Hoca, Zafer 160, Kaya and Bornova 92. This regenerable callus could be described as a callus with multiple shoot buds formed in a period of 3–4 weeks (Fig. 2C). Shoot buds could be maintained for a long culture period but they remained short. This phytohormone also lead to somatic embryogenesis as could be seen in Zafer 160 and Vamık Hoca (Fig. 2D). Somatic embryos did not proceed to form plantlets when separated and cultured.

Short primary root-like structures were also observed on calli incubated on ZR. Twenty of calli from four varieties (Akhisar, Kaya, Bornova 92 and Tokak) were transferred to TDZ medium after precultivation in dicamba (4 mg l^{-1}). Primary effect of TDZ was the formation of adventive buds and green solid sectors on calli as mostly seen in Akhisar. Bornova 92 and Kaya did not respond to TDZ treatment while 6

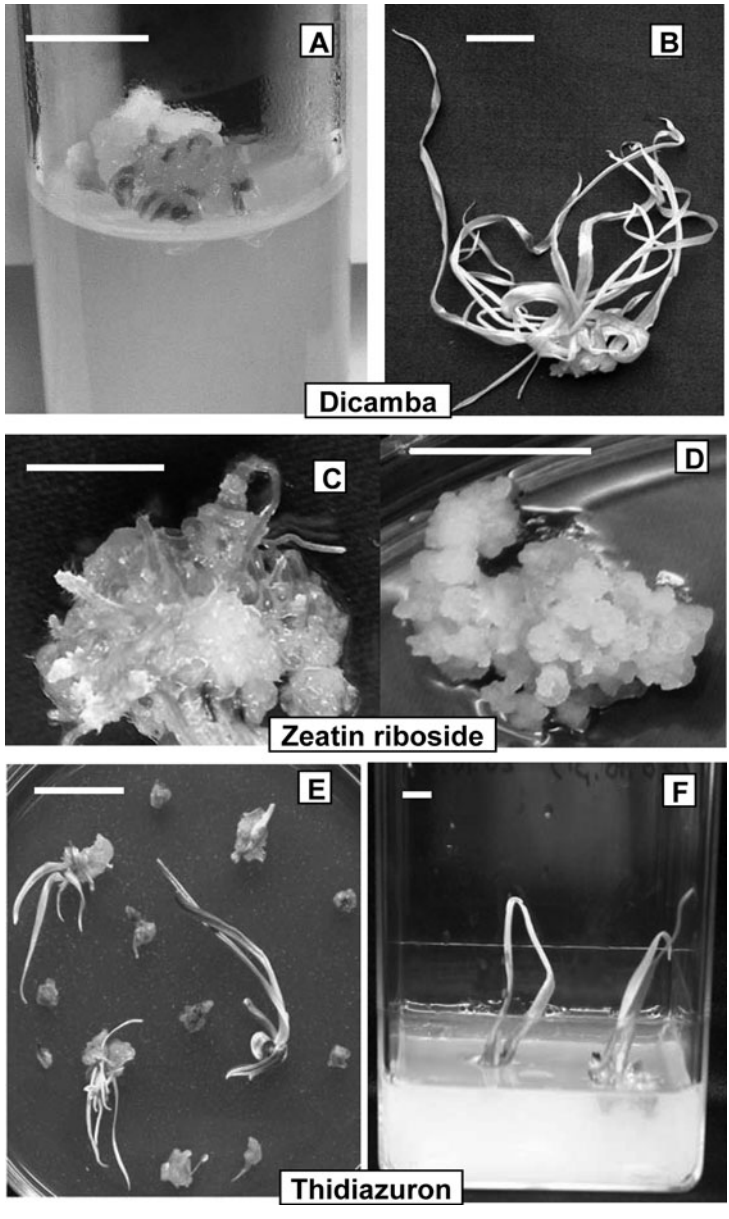

Fig. 2. Effects of different plant growth regulators (PGRs) on mature embryo-derived callus. A. Shoot induction MS + 0.5 mg/L dicamba (Bornova 92) after 1 week. B. Multiple shoots generated on callus incubated on MS + 0.5 mg/L dicamba (Bornova 92). C. Regenerable callus formation in Vamık Hoca on MS + 2 mg/L zeatin riboside. D. Somatic embryogenesis on callus (Zafer 160) incubated on MS + 2 mg/L zeatin riboside. E. Green solid sectors and adventitious buds on callus incubated on MS+2 mg/L thidiazuron (Akhisar). F. Plantlet formation from green sectors shown in E on TDZ medium (Akhisar). Bars represent 10 mm

calli from Tokak formed green sectors on the callus surface. In the case of negative effect of TDZ, callus color turned into black and followed by drying. The 2–3 green sectors per callus were obtained in Akhisar variety. When these compact green sectors were separated and transferred to the same media of 2 mg l^{-1} TDZ, shoot elongation (one/multiple) could be achieved from some of the explants (Fig. 2E). Total 51 green sectors were subcultured on fresh media and some of them exhibited shoot elongations (Fig. 2F). Finally 5 plantlets were recovered from green sectors on MS medium (w/o hormone).

DISCUSSION

This is the first report concerning the Turkish commercial varieties of barley for the assessment of their potentials in callus induction and regenerable callus formation. Use of MS salts with dicamba resulted in rapidly proliferating callus induction in all genotypes. We tested two kinds of auxins on primary callus induction from mature embryos. 2,4-D is a widely used auxin for callus induction in barley. However, its use may not be efficient in all barley genotypes from different geographical origins [2]. All varieties responded to the 2,4-D and dicamba treatments applied and the higher amounts of auxins (> 2 mg l^{-1}) were found to be more efficient in callus induction. This finding showed that barley needs higher auxin levels for callus formation unlike some wheat varieties [5]. Dicamba was effective in inducing of vigorously growing callus production in comparison with 2,4-D. Particularly, Bornova 92 and Tokak varieties were the most responsive genotypes in dicamba treatments. The use of dicamba alone or combined with 2,4-D also induced regenerable callus induction in European barley varieties [20]. Dicamba is not a common auxin used in barley tissue culture even though the positive effects of this compound on callus induction were demonstrated in early studies [16]. Akula et al. [2] did not find significant differences in callus formation induced by 2,4-D and dicamba when they investigated 9 barley varieties. There are also opposite results such as American variety "Morex" which showed higher callus induction in 2,4-D instead of dicamba [8].

Two types of callus (A and B) were observed in our work in different ratios. The only exception was Akhisar (with high numbers of type A callus) variety. Compact and nodular callus (referred as type B) kept growing vigorously in longer culture periods. On the contrary, type A callus was more sensitive than type B in phytohormone treatments and turned into brown color during long culture periods. As a result, morphogenic callus formation was genotype-dependent at defined media compositions and affected the regeneration potential of callus cells.

Even though the differentiation on the primary callus was different on each phytohormone treatments, thidiazuron (TDZ) and dicamba were the most effective growth regulators in stimulating shoot formations over callus. Zeatin riboside induced multiple shoots in a short time, out those shoots did not elongate (Fig. 2C). Use of this cytokinin in our work was effecient for producing somatic embryos in some varieties.

Influence of 0.5 mg l^{-1} dicamba on callus regeneration was efficient and resulted in multiple shoot formation. Multiple shoots were also seen in Kaya, Bornova 92 and Süleyman Bey varieties by longer dicamba treatments. Multiple primary roots were observed on calli of Süleyman Bey, Şerife Hanım and Bornova 92 (data not shown).

Precultivation with higher auxin concentrations (4 mg l^{-1}) slightly reduced the numbers of callus with shoots (Table 2). Callus that did not form shoots on regeneration medium was keeping proliferation. This may be explained by the suppression of cell differentiation in higher auxin concentrations. This effect was also observed on shoot elongation which was reduced in 4 mg l^{-1} dicamba precultivation (maximum 3.5 cm). Therefore, the use of lower auxin concentrations (< 4 mg l^{-1}) on precultivation may facilitate callus regeneration. Much lower concentrations of dicamba (0.02 – 0.1 mg l^{-1}) enhanced shoot numbers per callus on wheat calli [5]. Consequently, the use of dicamba is favourable to induce shoots instead of cytokinins. Formation of regenerable callus by 0.5 mg l^{-1} dicamba in only some of the varieties confirmed the idea of genotype dependency.

Use of TDZ for callus regeneration had different effects when compared to dicamba and ZR. Minimum 2–3 green compact sectors per callus appeared in 6–7 days on TDZ medium (Fig. 2E). However, the response to TDZ treatment was quite specific among the tested varieties. We could obtain regenerable callus with TDZ only in Akhisar (Fig. 2E). Calli from Kaya and Bornova 92 did not produce green sectors even though they grew vigorously on the callus induction medium. Subsequently shoot elongation was obtained with TDZ (Fig. 2F). This compound showed both auxin and cytokinin activities on differentiation [18] and was mostly used in dicotyledonous species for promoting callus regeneration. Even though it is not widely used in cereals, multiple shoot induction from barley mature embryos has been reported by TDZ without a callus phase [10]. Thidiazuron seemed to have a potential for callus regeneration of barley. However, further optimizations considering its concentration and combination with other growth factors may be necessary.

Major regeneration systems are based on immature embryo derived cultures in barley. Recently, mature embryos have also been preferred as a starting material for the purpose of *in vitro* plant regeneration. Our work demonstrated that Tokak and Bornova 92 were quite responsive to the tested auxins by efficient callus induction. Development of vigorously growing callus cultures could be obtained in 7–8 days from mature embryos by using dicamba in MS salts. Overall data demonstrated that regeneration on barley callus can be induced by either TDZ or lower dicamba concentrations.

Calli derived from mature embryos are not preferred as targets for DNA transfer because of their low differentiation capability. However, previous work with different barley genotypes demonstrated that the efficient use of mature embryos is possible depending on the selected genotypes and setting up the optimal tissue culture conditions [2, 10]. Regeneration units (green sectors, etc.) obtained from undifferentiated callus cells using appropriate media can be ideal targets for transformation studies.

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