ISSN 1479-2621
Plant Genetic Resources: Characterization and Utilization (2017) **15**(1); 45–50
doi:10.1017/S1479262115000350

Biotechnological characterization of a diverse set of wheat progenitors (*Aegilops* sp. and *Triticum* sp.) using callus culture parameters

Murat Özgen*, Melahat A. Birsin and Berk Benlioglu

Department of Field Crops, Agriculture Faculty, Ankara University, 06110 Diskapi, Ankara, Turkey

Received 20 April 2015; Accepted 26 June 2015 – First published online 14 August 2015

Abstract

It is known that genetic diversity is the most important factor in classical and modern plant breeding. The considerable increase in the number of transgenic crops reveals the value of new plant genetic resources. In this study, a set of 12 wheat progenitors were screened for tissue culture parameters such as callus induction, callus weight, regeneration capacity of callus and callus efficiency using mature embryos. Embryos were excised from imbibed seeds of the progenitors. The excised embryos were placed scutellum upwards in dishes containing 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) for callus induction. The developed calli and regenerated plants were maintained on 2,4-D free MS medium. When mature embryos of 12 wheat progenitors (*Aegilops* sp. and *Triticum* sp.) were compared, significant differences were detected in callus induction frequency, weight of callus, regeneration capacity and culture efficiency. A significant genotypic effect was observed on the culture responses. Of the 12 wheat progenitors tested, *Aegilops umbellulata* had the highest regeneration capacity of callus. *Aegilops biuncialis* created the most regenerable calli because of the highest callus induction and culture efficiency. In the experiment, callus induction was significantly correlated with callus weight ($r = 0.820$) and regeneration capacity ($r = 0.955$). Weight of callus was significantly correlated with regeneration capacity ($r = 0.740$), while there was no significant correlation between callus induction frequency and culture efficiency ($r = 0.350$). Our results showed that, generally, mature embryos of some *Aegilops* and *Triticum* species have a high regeneration capacity, and therefore, can be used as an effective explant source for the successful application of biotechnology in crop improvement.

Keywords: *Aegilops*; callus culture; genetic resource; mature embryos; *Triticum*; wheat progenitors

Introduction

Wheat progenitors are often used as sources of desirable agronomic characters that could be introduced into cultivated new varieties. It has long been known that *Aegilops* are resistant to various wheat diseases of which most important are rusts (*Puccina* spp.) (Özgen, 1987) and to salinity (Colmer *et al.*, 2006). Especially, *Aegilops umbellulata* (Özgen, 1983), *Aegilops triaristata* (Özgen, 1985) and *Aegilops biuncialis* (Özgen, 1984; Damania and Pecceti, 1990) are resistant to yellow rust. *Ae. Biuncialis* is also resistant to brown rust (Dimov, 1993). On the other hand, *Triticum monococcum* (Datta *et al.*, 1995), *Ae. biuncialis* (Gorham, 1990), *Aegilops cylindrica* (Farooq *et al.*, 1989; Gorham, 1990), *Aegilops triuncialis* (Farooq *et al.*, 1989), *Ae. umbellulata* (Gorham, 1990)

* Corresponding author. E-mail: ahmetmuratozgen@gmail.com

contain genes for salt tolerance. Therefore, plant genetic resources are necessary to food security for the future. Genetic resources are usually considered as a public good and shared internationally. Wild relatives of crop species represent the store of genetic diversity that will help to provide the food requests for billions of people by 2050. New technologies from genomics support classical plant breeding for enhancing traits to meet these food requests. Genetic diversity is the lifeblood of all kinds of plant breeding. The increase in the number of biotech crops reveals the value of global food security (Philip, 2014).

Successful plant regeneration from cultured tissues is required for the application of biotechnology in crop improvement. The achievement of tissue culture depends upon appropriate culture techniques and genotypes. The tissue culture parameters such as callus induction and plant regeneration of wheat are generally affected by the genotype (Sears and Deckard, 1982; Chowdhury *et al.*, 1991; Fennel *et al.*, 1996; Özgen *et al.*, 1998; Bi *et al.*, 2007), explant source (Ozias-Akins and Vasil, 1982; Zhang and Seilleur, 1987; Redway *et al.*, 1990), and culture medium (Mathias and Simpson, 1986; Fennel *et al.*, 1996; Barro *et al.*, 1999; Mendoza and Kaeppler, 2002; Satyavathi, 2004).

Immature embryos were shown to be the most suitable explant for callus induction and plant regeneration via *in vitro* cultivation of wheat (Maddock, 1985; Tuberosa *et al.*, 1988; Redway *et al.*, 1990; Ke *et al.*, 1995; Tang *et al.*, 2004). Therefore, immature embryos have been used mostly as an explant in wheat tissue culture. Recently, it has also been used in molecular studies in wheat and its relatives (Zhao *et al.*, 2014). However, it is usually difficult to obtain immature embryos throughout the year, and their appropriate stage for culture is also limited. Whereas, the use of mature embryos reduces greenhouse costs, and saves space and time (Zale *et al.*, 2004). Mature embryos are also available in a large amount all year round.

Researches on the embryo culture of wheat progenitors are very inadequate. The purpose of this study was to screen a diverse set of wild wheat species (*Aegilops* sp. and *Triticum* sp.), which are very important progenitors of wheat cultivars, for their ability to induce callus and regenerate plants from mature embryos, for ultimate use in genetic transformation of wheat genotypes.

Materials and methods

Plant materials

A total of 12 accessions of *Aegilops aucheri* ($2n = 2x = 14$, BB), *Ae. Biuncialis* ($2n = 2x = 28$, $C^UC^UM^bM^b$), *Ae. cylindrica* ($2n = 2x = 28$, CCDD), *Ae. triaristata* ($2n = 2x = 28$, $C^UC^UM^tM^t$), *Ae. triuncialis* C^UC^UCC), *Ae. umbellulata* ($2n = 2x = 14$, C^UC^U), *Aegilops speltoides* ($2n = 2x = 14$, BB), *Aegilops ligustica* (2n = 2x = 14, BB), *Triticum monococcum* ($2n = 2x = 14$, A^mA^m), *Triticum dicoccum* ($2n = 2x = 28$, AABB), *Triticum spelta* ($2n = 2x = 42$, AABBDD), *Triticum vavilovii* ($2n = 2x = 42$, AABBDD) were used as sources of mature embryos. Dry seeds of these wheat progenitors were supplied from Prof. Dr M. Özgen, Ankara University, Faculty of Agriculture, Ankara, Turkey. For naming of the *Aegilops* and *Triticum* species, the classifications of van Slageren (1994) and Hammer *et al.* (2011) were taken as a base, respectively.

Callus induction and plant regeneration

Dry seeds were surface sterilized with 70% (v/v) ethanol for 5 min, washed several times with sterile distilled water, treated for 20 min with commercial bleach, and rinsed several times with sterile distilled water. The seeds were then imbibed in sterile water for 2 h at 33°C. Thereafter, mature embryos were aseptically dissected slightly with a scalpel from the endosperm of imbibed seeds for callus induction. The embryos were cultured, with the scutellum upwards, in sterile 10-cm Petri dishes containing the mineral salts of MS (Murashige and Skoog, 1962), 20 g/l sucrose, 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 7 g/l agar. The dishes were kept at 25 ± 1°C in total darkness for 14 d (method modified from Özgen *et al.*, 1996, 1998).

Embryogenic calli were then removed from the embryos and transferred onto a hormone-free shoot initiation medium containing MS mineral salts (Murashige and Skoog, 1962), glycine (2 mg/l), sucrose (20 g/l) and agar (7 g/l) in the plates. Callus fresh weight data were obtained before calli were transferred to shoot initiation medium in embryo cultures. The transferred callus cultures were incubated at 25 ± 1°C for 3 weeks in the dark, and then grown under 16 h/8 h (light/dark) photoperiod at 25 ± 1°C. At the end of 6–8 weeks, the frequency of differentiation for each species was calculated, based on the number of calli with green spots. Culture efficiency was based on regenerable calli out of the number of mature embryos cultured. When the regenerated shoots elongated to 10–15 mm, were transferred to Magenta boxes containing the same medium. Cultures for rooting were exposed to 50 W cool white fluorescent light (50 μmol/m^2/s) at 25 ± 1°C under 16 h/8h (light/dark) photoperiod for 3 weeks. The all media were adjusted to pH 5.8 and autoclaved for 20 min at 121°C and 1.1 kg/cm^2 pressure. Callus fresh weight data were obtained before calli were transferred

https://doi.org/10.1017/S1479262115000350 Published online by Cambridge University Press

to shoot initiation medium in embryo cultures (Özgen *et al.*, 1998).

Statistical analysis

The tissue culture parameters included the callus induction frequency, callus weight, regeneration capacity of callus (number of nodular calli with green spots/number of calli induced × 100), and culture efficiency (number of nodular calli with green spots/number of embryos cultured × 100). A completely randomized design with three replications per species was used. Petri dishes containing 15 seeds were considered the units of replication. The effects of species on culture responses were determined by analysis of variance and least significant difference tests. Correlation coefficients between the different characters were calculated for each procedure (Steele and Torrie, 1960).

Results

In the experiment, mature embryos from a diverse set of 12 wheat progenitors were tested for tissue culture parameters. The variables for this experiment included

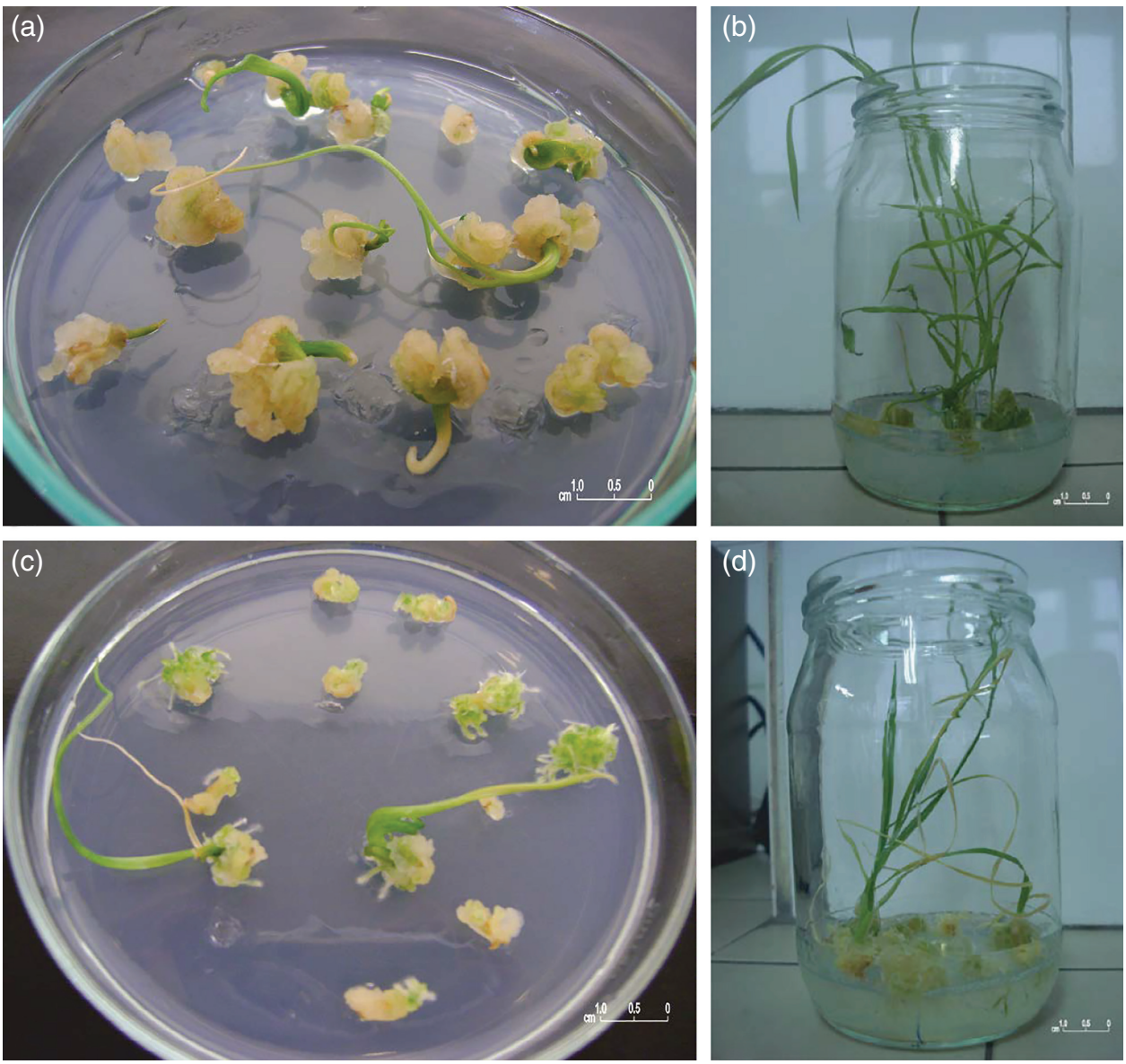

Fig. 1. Callus maintenance and plant regeneration from mature embryos of *Aegilops biuncialis* (a and b) and *Aegilops umbellulata* (c and d).

https://doi.org/10.1017/S1479262115000350 Published online by Cambridge University Press

Table 1. Mature embryo culture responses of *Aegilops* and *Triticum* species

Species[a]	Genome	Callus induction (%)	Weight of callus (g)	Regeneration capacity of callus (%)[b]	Culture efficiency (%)[c]
Aegilops aucheri	BB	17.7de[d]	0.260g	55.5d	8.9f
Aegilops biuncialis	$C^UC^UM^bM^b$	97.8a	0.880c	93.1ab	91.1a
Aegilops cylindrica	CCDD	15.5e	0.233g	55.6d	8.9f
Aegilops triaristata	$C^UC^UM^tM^t$	82.2b	1.247a	73.1bcd	60.0c
Aegilops triuncialis	C^UC^UCC	53.3c	0.407f	79.2abc	42.2de
Aegilops umbellulata	C^UC^U	46.6c	0.490ef	95.8a	39.9de
Aegilops speltoides	BB	44.4c	0.227g	88.9abc	40.0de
Aegilops ligustica	BB	26.6d	0.240g	91.7ab	26.7e
Triticum monococcum	A^mA^m	91.1ab	0.810c	69.5cd	55.5cd
Triticum dicoccum	AABB	97.8a	1.067b	82.0abc	80.0ab
Triticum spelta	AABBDD	93.3a	0.570de	76.7abcd	71.1bc
Triticum vavilovii	AABBDD	88.8ab	0.657d	92.5ab	82.2ab
Mean ± SE		55.6 ± 9.73	0.590 ± 0.10	79.5 ± 4.05	50.5 ± 7.98
$LSD_{0.05}$		9.9	0.09	21.2	15.8

[a] The origin of the species is Turkey. [b] No. of regenerable calli (nodular calli with green spots)/no. of calli induced × 100. [c] No. of regenerable calli/no. of embryos cultured × 100. [d] Means followed by the different letters are significantly different at the 0.05 probability level.

callus induction frequency, weight of callus, regeneration capacity of callus, and culture efficiency.

Callus formation from embryos started after 2–3 d of culture. On the 6th day, the average diameter of the calli was 5–6 mm. Maximum callus induction occurred 14 d after placement on the mature embryo callus media. Calli with green spots rapidly developed shoots and leaves in shoot initiation medium. These plantlets did not establish an adequate root system when 2,4-D was present in the medium. Following transfer to 2,4-D-free MS medium, plants regenerated and produced multiple shoots from mature embryo calli (Fig. 1(a)–(c)). After about 3 months from callus initiation, regenerated plants (with shoot and root) were obtained in a jar (Fig. 1(b)–(d))

Culture responses were greatly influenced by the species for embryo culture (Table 1). On the other hand, a significant correlation was also observed between callus fresh weight and callus induction ($r = 0.820$, $P < 0.01$) or culture efficiency (0.740, $P < 0.01$) (Table 2). Therefore, frequencies of callus induction and regeneration capacity of callus of 12 wheat progenitors were significantly different. Callus induction frequency was highest in *Ae. biuncialis* (97.8%), *T. dicoccum* (97.8%), *T. spelta* (93.3%) and lowest in *Ae. cylindrica* (15.5%), *Ae. aucheri* (17.7%) and *Ae. ligustica* (26.6%) (Table 1). On the other hand, *Ae. umbellulata* (95.8%), *Ae. biuncialis* (93.1%), *T. vavilovii* (92.5%) and *Ae. ligustica* (91.7%) had an excellent regeneration capacity of callus, while *Ae. aucheri* (55.5%) and *Ae. cylindrica* (55.6%) had lowest regenerable callus frequencies (Table 1).

Discussion

The effect of genotype on the regeneration of callus from mature embryos in wheat progenitors was similar to other wheat studies (Özgen *et al.*, 1996; Özgen *et al.*, 1998; Tan and He, 2001; Ren *et al.*, 2003; Yu *et al.*, 2003).

Table 2. Correlations between different characters in callus cultures from mature embryos of *Aegilops* and *Triticum* species

	Correlation coefficients between characters			
Characters	1	2	3	4
Callus induction (%)	–	0.820**	0.350	0.955**
Weight of callus (g)		–	0.128	0.740**
Regeneration capacity of callus (%)			–	0.541
Culture efficiency (%)				–

** Significantly different from zero at $P = 0.01$ probability.

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On the other hand, it showed that culture efficiency was highest in *Ae. biuncialis*, *T. vavilovii* and *T. dicoccum* which all showed a better tissue culture response than the other genotypes (Table 1). These results showed that the genotype effect and genotype dependency were also high in the tissue culture of mature embryos from wheat genitors. *Ae. biuncialis* had both high callus induction (97.8%) and regenerable calli frequencies (93.1). Therefore, *Ae. biuncialis* had the most frequency of culture efficiency (91.1%) in the studied wheat progenitors. Such species are very desirable in tissue culture and plant breeding programmes.

Frequencies of callus induction were generally high but there was no significant relationship with the regeneration capacity of callus in the studied wheat progenitors (Table 2). Although some genotypes such as *Ae. biuncialis* had both high callus induction and plant regeneration capacity, the correlation coefficients between callus induction frequency and regeneration capacity or between regeneration capacity and culture efficiency were statistically insignificant (Table 2). Generally, it is known that callus induction and regeneration capacity may be controlled independently of each other (Sears and Deckard, 1982; Chowdhury *et al.*, 1991; Özgen *et al.*, 1996). The absence of significant relationships between callus induction frequency and regeneration capacity of callus clearly confirms that these characters are genetically independent. Thus, *T. dicoccum* (97.8%) and *T. spelta* (93.3%) had a high callus induction frequency but a low regeneration capacity of callus (82.0 and 76.7%, respectively), while *Ae. ligustica* (26.6%) and *Ae. umbellulata* (46.6%) had a low callus induction frequency but most calli were regenerable (91.7 and 95.8%, respectively) in the embryo culture (Table 1).

Genotype effects on plant regeneration from wheat embryo cultures have been reported previously (Sears and Deckard, 1982; Tuberosa *et al.*, 1988). These results suggested that the plant regeneration was independent on the other characters. It is known that, no significant correlations existed among the studied tissue culture parameters suggesting that different genes or gene combinations may control such responses (Özgen *et al.*, 1998). However, a significant correlation between callus induction frequency and culture efficiency ($r = 0.955$, $P < 0.01$) in mature embryo culture indicated that culture efficiency tended to increase with increasing callus induction rate (Table 2). This result was consistent with the reports of Özgen *et al.* (1996), Özgen *et al.* (1998) and Zale *et al.* (2004).

This research has demonstrated that some species of wheat progenitors such as *Ae. umbellulata* and *Ae. biuncialis* can induce callus and regenerate plants from mature embryos at high frequencies. While immature embryos and immature inflorescences are an excellent source of explant for regenerating large numbers of plants, mature embryos are also an important source and can thus be used to save the time and growth space required to grow plants to post anthesis for embryo collection in wheat progenitors. To increase the plant regeneration capacity in mature embryo cultures of wheat progenitors, therefore, suitable species should be used. Mature embryos may also reduce some of the difficulties associated with obtaining explants of immature embryos and immature inflorescences for tissue culture of wheat progenitors.

In conclusion, there were strong genotypic influences on callus induction, callus weight, regeneration capacity and plant regeneration in mature embryo culture of wheat progenitors. There was no significant correlation between callus induction frequency and regeneration capacity of callus. These results suggest that regeneration capacity of callus was a key step in the tissue culture of wheat progenitors. The present study also showed that culture responses of mature embryos from wheat progenitors might be more useful than the other explants. The transfer of foreign genes into wheat by genetic engineering techniques requires the development of efficient *in vitro* regeneration systems. An efficient regeneration system such as the mature embryo culture may provide enough material for gene isolations and direct gene transfer studies in wheat progenitors. Therefore, mature embryos, which are readily available throughout the year, can be used as an effective explant source in the tissue culture of wild wheat species.

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