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Study on Plant Regeneration of Wheat Mature Embryos Under Endosperm-Supported Culture

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Abstract

To reveal the suitability of using mature embryos as an explant source in wheat tissue culture, mature embryos from eight common wheat cultivars (*Triticum aestivum* L. *cv.*) were cultured with or without endosperm to test their efficiency of callus induction and plant regeneration. When embryos were cultured together with endosperm (endosperm-supported culture, ES), the percentage of callus induction was significantly lower than that when embryos were cultured in the absence of endosperm (non-endosperm-supported culture, NES). This pattern was evident in most genotypes, regardless of whether 2 or 8 mg L^{-1} 2,4-D was added in the NES culture. However, in ES culture, more induced calli were differentiated into distinct green spots and they further developed into plantlets. Thus, more plants were regenerated in ES culture than in the NES treatment. Most of the eight tested genotypes showed a significant difference in callus induction rate and plantlet regeneration in both ES and NES cultures. In addition, the enzymatic activity of oxalate oxidase in the callus of ES culture condition was obviously higher than that in the callus of NES culture condition, suggesting that the activity of oxalate oxidase may be a parameter for selection of calli with potential for plantlet regeneration. These results indicate that wheat mature embryos are valuable explants for highly efficient callus induction and plant regeneration, if proper treatment and medium are used.

Key words: mature wheat embryo, tissue culture, endosperm-supported, plant regeneration, oxalate oxidase activity

INTRODUCTION

Genetic transformation is a powerful tool that has been used extensively in crop improvement and plant molecular biology study, including agriculturally important crops such as wheat, rice, and maize. However, the genetic transformation of wheat is severely retarded, compared to the progress achieved in rice and maize. One of the biggest obstacles is the low efficiency of plant regeneration in wheat tissue culture. It is well known that the frequencies of callus induction and plant regeneration in wheat tissue culture completely depend on medium composition (Lazar *et al.* 1983; Mathias and Simpson 1986; Fennel *et al.* 1996) and explant sources (Ozias-Akins and Vasil 1982; Maddock *et al.* 1983; Zhang and Seilleur 1987; Redway *et al.* 1990). It is commonly regarded that the immature embryo (Maddock 1985; Redway *et al.* 1990; Tuberosa *et al.* 1988) is the best explant source for tissue culture among all explants, including inflorescence (Ozias-Akins and Vasil 1982; Maddock *et al.* 1983; Redway *et al.* 1990; Sharma *et al.* 1995), mesocotyl (Yurkova *et al.* 1982), seed (Gosch-Wackerle *et al.* 1979), immature leaves (Ahuja *et al.* 1982; Zamora and Scott, 1983), apical meristem (McHugen 1983), mature embryo (Ozias-Akins and Vasil 1983; Heyser *et al.* 1985; Chowdhury *et al.* 1991), and thin mature embryo fragments (Delporte *et al.* 2001). However, immature em-

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bryos have some disadvantages as explants. For example, their availability is strictly limited in a sharp period of growing time in the year, and the suitable culturing stage is extremely difficult to discriminate. Because of the advantages such as easy storage and ready availability at all times throughout the year, mature embryos as a favorable explant source were explored broadly in wheat tissue culture. The main bottleneck with mature embryo as explant is low plant regeneration. This made them difficult to use in practice. To improve mature embryo culture efficiency, endosperm-supported (ES) culture method was explored (Ozgen *et al.* 1998). Unfortunately, few experiments have been reported since then, and therefore its reliability and universality need to be confirmed further.

To this effect in the present experimental paradigm, two methods, non-endosperm-supported (NES) and ES with different 2,4-D concentration supplements in media were applied to study the efficiency of common wheat mature embryos in culture. At the same time, activities of oxalate oxidase, a key enzyme in modifying cell wall mechanical properties that regulate the formation of embryogenic calli, were analyzed in the calli derived from different treatments. The goal was to verify the validity and practicality of ES culture method for the growth of cultivars with different genetic background and explore the possibility of oxalate oxidase activity as a reliable index to discriminate embryogenic and non-embryogenic calli.

MATERIALS AND METHODS

Plant materials

The eight winter wheat varieties, *Triticum aestivum* L. *cv.*, namely, Jimai 1, Guomai 1, Guoyou 1, Yumai 18, Yumai 34, Yumai 36, Xinmai 13, and Neixiang 188, were used as the source of mature embryos. The seeds used in this experiment were harvested from the experimental farm of Henan Agricultural University in Zhengzhou, China in June 2004, desiccated, and stored in desiccators at room temperature.

Tissue culture

For surface sterilization, mature seeds were soaked in 75% (v/v) ethanol for 5 min, washed four times with sterile distilled water, and then treated with 0.1% $HgCl_2$ (w/v) containing drops of Tween-20 for 30 min. The seeds were rinsed several times using sterile distilled water, and then were imbibed in sterile water at 33°C for 2 h. For callus induction, two culture methods were used: NES and ES. In ES callus induction treatment, the mature embryos of the imbibed seeds were aseptically loosened slightly with a scalpel. The seeds with their embryos still in contact with endosperm were then placed in sterile dishes containing 2,4-D (8 mg L^{-1}) and agar (7 g L^{-1}) with the furrows downward. In NES callus induction treatment, the mature embryos were excised aseptically from the imbibed seeds, and were placed subsequently, with the scutellum upward, on a solid agar medium containing the mineral salts used in the method of Murashige and Skoog (1962), 20 g L^{-1} sucrose, and 7 g L^{-1} agar. Two or eight milligram per liter of 2,4-D was supplied in the media, respectively. The dishes were kept at 25±1°C in total darkness. After 11 days of culturing, the plantlets that germinated directly from mature embryos were cut off and the developing calli were transferred onto a hormone-free shoot initiation medium containing MS mineral salts, glycine (2 mg L^{-1}), sucrose (20 g L^{-1}), and agar (7 g L^{-1}). After 3 weeks of culture in darkness, the calli were transferred onto a regeneration medium. The transferred calli were incubated at 25±1°C and with a 16-h photoperiod (30 μmol m^{-2} s^{-1}). After 3 weeks, green spots could be observed in calli. Those calli with green spots were subcultured to form plantlets, which were transferred to flasks containing the same medium. After 30 days, the developed plantlets were transferred directly from the regeneration medium to pots containing soil for further rooting. Plantlets were maintained at 25±1°C and with a 16-h photoperiod for 1 week (about 18 mol m^{-2} total light intensity). After 3 weeks of growth, the plants were vernalized by exposure to a low temperature of 0-4°C and a 16-h photoperiod for 4 weeks. They were then kept in a greenhouse till maturation.

The data were obtained as follows:

Callus induction efficiency = (The number of calli ÷ The number of mature embryo) × 100%

Green spot differentiation efficiency = (The number of calli with green spots ÷ The number of calli) × 100%

Plants regeneration efficiency = (The number of plants regenerated ÷ The number of mature embryos) × 100%

Culture efficiency = Callus induction efficiency × Green spot differentiation efficiency × Plants regenerated efficiency × 100%

A random design with three replications per genotype was used. The effect of genotypes on culture efficiency was evaluated by the SPSS software. Correlation coefficients between the different characters were determined for each procedure. Differences between ES and NES were assessed using the analysis of variance and least significant difference tests.

Oxalate oxidase assay

Oxalate oxidase activity was detected using a modified procedure of Lane *et al.* (1993) from the method of Sugiura *et al.* (1979). The main steps were as follows: Calli were collected from mature embryos of Yumai 18 after culture for 11 days and were homogenized in an Eppendorf tube with 100 μL succinate buffer (pH 3.5). The homogenates were centrifuged at 12 000 × g for 5 min and the pellets were resuspended in another 100 μL of fresh buffer with or without (in control samples) 2 mM oxalic acid. The mixture was incubated at 25°C for 10-30 min before adding 60 μL developer (Sugiura *et al.* 1979), and then given a further incubation for 10 min. Homogenates were centrifuged and the absorbance of supernatants was detected at 550 nm. The product was quantified by reference to a set of standard concentration of hydrogen peroxide. The concentration of protein in each Eppendorf after the first centrifugation was determined using the BioRad protein assay reagent.

RESULTS

Callus induction

The callus growth from the mature embryos in ES and NES treatments started almost at the same time, about 2-3 days after culture. The optimum sizes (about a half seed) of calli from mature embryos for subculturing occurred after 11 days of culture in the callus induction media (Fig.-A and D).

After 3 weeks of culture in darkness, the effect of different treatments and genotypes on the induction of callus during the mature embryo culture was observed (Table 1). The NES culture condition consistently and significantly promoted the callus induction when either 2 or 8 mg L^{-1} 2,4-D was added in the medium compared to ES culture of all genotypes (82.7%/82.1% vs. 52.3%). Different genotypes showed significant difference in the ability of callus induction in both ES and NES culture. For example, under the ES culture, the average frequency of callus induction in all tested cultivars was 52.3%, the highest (89.6%) in Jimai 1 and Yumai 18, and the lowest (11.6%) in Yumai 34. In addition, most of the genotypes showed approximately the same ability of callus induction in different cultures. For example, Yumai 36 always had the lowest percentage of callus induction in all three cultures and Yumai 18 the highest (Table 1). There was no apparent difference in the average frequency between 2 and 8 mg L^{-1} 2,4-D concentrations in NES culture.

In general, two types of calli (non-embryogenic calli and embryogenic calli) were observed after 3 weeks. Non-embryogenic calli were characterized by a cream color and a soft watery texture, whereas embryogenic calli were pale, smooth, compact, regenerable, and contained embryonic structures that occurred as either independent or fused nodules. Except for Yumai 34 and Neixiang 188, which possessed embryogenic calli, all other genotypes produced non-embryogenic calli.

Callus differentiation and plantlet regeneration

After 3 weeks of culture in darkness, the calli were transferred onto a regeneration medium. About 1 week later, green spots started to appear on the surface of calli. After 3 weeks of culture and after three treatments, eight genotypes showed differences in their green spot differentiation (Table 1, Fig.-B and E). In the ES culture, the average efficiency was 62.3%, the highest in Yumai 34, 83.0%, and the lowest in Xinmai 36, 49.0%. In the NES culture, there were two treatments regarding 2,4-D concentration. When the higher 2,4-D concentration (8 mg L^{-1}) was used for culturing, the average green spots efficiency was 36.1%, the highest in Yumai 34, 57.3%, and the lowest in Jimai 1, 23.5%. Comparing with the

higher concentration treatment, it was observed that the green spots efficiency at the lower 2,4-D concentration (2 mg L^{-1}) was obviously higher, the average was 48.8%, the highest in Yumai 34, 73.1%, and the lowest in Jimai 1, 37.3%. Among all the tested cultivars in different treatments, Yumai 34 had the highest efficiency.

Callus with green spots rapidly developed shoots and roots on the initiation medium (Fig.-C). Plant regeneration efficiency was dramatically affected by the different treatments and genotypes (Table 1). In the ES culturing condition, plant regeneration efficiency averaged 43.7%, varying from 25.0% in Jimai 1 to 65.8%

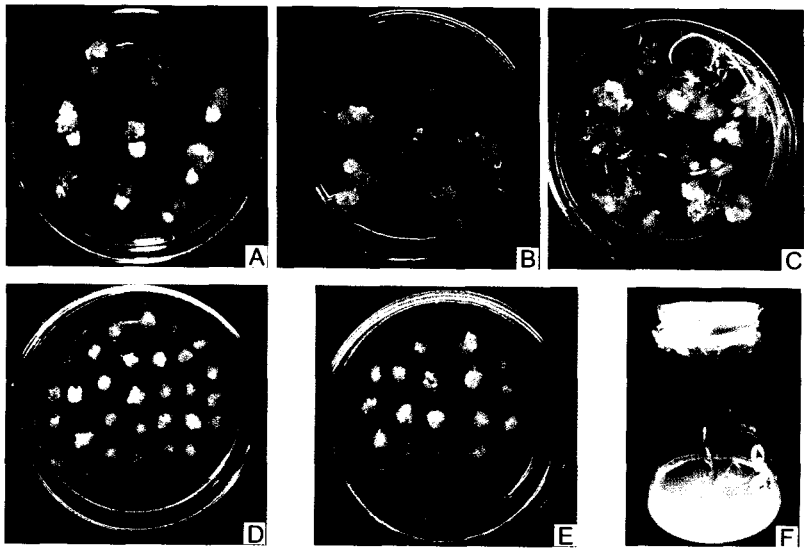

Fig. Callus induction, differentiation from mature embryos, and plantlets regeneration. A, 11-d calli by ES culture; B, 45-d calli by ES culture (with green spots and shoots); C, plantlets regenerated from ES culturing calli; D, 11-d calli by NES culture; E, 45-d calli from NES culture (with green spots); F, a vernalized plantlet.

Table 1 The effect of different treatments on callus induction and differentiation from mature wheat embryos

Genotype	Treatment	2,4-D (mg L^{-1})	Callus induction (%)	Callus with green spots (%)	Regenerated plantlet (%)	Culture efficiency (%)
Jimai 1	ES	8	89.6 a	82.2 b	25.0 h	18.4 c
Guomai 1	ES	8	24.3 f	54.6 g	65.8 a	8.7 e
Guoyou 1	ES	8	60.1 c	60.1 d	60.0 b	21.7 a
Yumai 18	ES	8	89.6 a	57.6 e	39.5 e	20.4 b
Yumai 34	ES	8	11.6 g	83.0 a	54.3 c	5.2 g
Yumai 36	ES	8	45.4 d	49.0 h	29.3 g	6.5 f
Xinmai 13	ES	8	64.4 b	57.4 f	32.7 f	12.1 d
Neixiang 188	ES	8	32.4 e	62.1 c	43.2 d	8.7 e
Mean	**ES**		**52.3±27.7 B**	**62.3±12.0 A**	**43.7±14.3 A**	**12.7±6.2 A**
Jimai 1	NES	8	100.0 a	23.5 f	5.6 h	11.3 f
Guomai 1	NES	8	91.9 c	28.7 d	12.5 c	3.3 b
Guoyou 1	NES	8	100.0 a	36.4 c	18.3 b	6.7 a
Yumai 18	NES	8	100.0 a	35.4 c	7.7 f	2.7 c
Yumai 34	NES	8	22.4 e	57.3 a	18.7 a	2.4 d
Yumai 36	NES	8	97.1 b	25.8 e	8.6 e	2.1 e
Xinmai 13	NES	8	100.0 a	40.1 b	6.7 g	2.7 c
Neixiang 188	NES	8	45.6 d	41.8 b	12.0 d	2.3 d e
Mean	**NES**		**82.1±29.1 A**	**36.1±10.3 C**	**11.2±4.8 C**	**2.9±1.5 C**
Jimai 1	NES	2	89.4 e	37.3 h	20.7 e	6.9 e
Guomai 1	NES	2	96.4 b	52.8 b	26.7 a	13.6 a
Guoyou 1	NES	2	94.1 c	50.2 c	23.1 d	10.9 b
Yumai 18	NES	2	100.0 a	42.3 f	9.2 g	3.9 f
Yumai 34	NES	2	40.8 g	73.1 a	24.1 c	7.2 d
Yumai 36	NES	2	100.0 a	39.5 g	25.0 b	9.9 c
Xinmai 13	NES	2	91.8 d	44.3 e	8.0 h	3.3 g
Neixiang 188	NES	2	50.0 d	50.0 d	15.7 f	3.9 f
Mean	**NES**		**82.7±22.5 A**	**48.8±10.8 B**	**19.1±6.9 B**	**7.4±3.6 B**

Genotype differences followed by small letters are marked at the 0.05 probability level. Mean differences followed by capital letters are marked at the 0.05 probability level.

in Guomai 1. In the NES culturing condition, the effect of lower 2,4-D concentration (2 mg L^{-1}) on plant regeneration was better than that of the higher concentration (8 mg L^{-1}). The result was the same for induction of green spots. At 8 mg L^{-1} 2,4-D concentration, the plantlets' regeneration efficiency averaged 11.2%, with the highest (18.7%) in Yumai 34 and the lowest (5.6%) in Jimai 1. At 2 mg L^{-1} 2,4-D level, Guomai 1 had the highest efficiency (26.7%), and Xinmai 13 had the lowest (8.0%), and the average in the 8 genotypes was 19.1%. The result suggested that the high 2,4-D concentration might have had a negative effect on callus differentiation in NES culturing condition.

Culture efficiency

Significant difference in culture efficiency among the different genotypes and treatments was also observed (Table 1). For example, under the ES culturing condition, the average culture efficiency was 12.7%. On the other hand, under the NES culturing condition, the average culture efficiency was 2.9% (8 mg L^{-1} 2,4-D) and 7.5% (8 mg L^{-1} 2,4-D), respectively.

Correlations among the callus induction, green sport differentiation, and plantlet regeneration

Correlation among the percentages of the callus induction, green sport differentiation, and plantlet regeneration in different treatments was analyzed (Table 2). The results suggested that there were some or significant negative correlations between callus induction and green spot differentiation and between callus induction and regeneration. In fact, callus quality was essential for callus differentiation regardless of callus induction efficiency. For example, Jimai 1 had a high efficiency of callus induction, but most of their calli were non-embryogenic, i.e., they had a loose and watery texture and lacked the ability to regenerate plants. Yumai 34, which had a low efficiency of callus induction, showed a higher efficiency of plant regeneration (Tables 1 and 2).

Table 2 Correlations among the callus induction, green sport growth, and plantlet regeneration[1)]

Character	ES[2)]			NES[2)]			NES[3)]		
	(1)	(2)	(3)	(1)	(2)	(3)	(1)	(2)	(3)
(1)		1		1		1			
(2)	-0.015	1	-0.813*	1	-0.740*	1			
(3)	-0.594*	-0.072	1	-0.586*	0.611*	1	-0.0.95	0.342	1

[1)] (1) callus induction efficiency; (2) green spots efficiency; (3) plant regeneration efficiency.
[2)] 8 mg L^{-1} 2,4-D.
[3)] 2 mg L^{-1} 2,4-D.
* Significant difference at the 0.05 probability level.

Oxalate oxidase activity

Oxalate oxidase activities in different calli (11 days) were represented by the accumulation of their reaction products (H_2O_2) that fuels the peroxidase-linked colorimetric reaction in the presence of exogenous oxalate (Table 3). The enzymatic activity of oxalate oxidase in calli (Yumai 18) derived from the ES treatment was obviously higher than that in calli from the NES treatments. Oxalate oxidase activity at low 2,4-D (2 mg L^{-1}) concentration was slightly higher than that at high 2,4-D (8 mg L^{-1}) concentration in the NES treatment.

Table 3 The effect of different treatments on oxalate oxidase activity in calli from mature embryos (Yumai 18)

Treatment	2,4-D (mg L^{-1})	Activities (nmol H_2O_2 mg^{-1} min^{-1})
ES	8	2.830 ± 0.0004 a
NES	8	2.373 ± 0.003 c
NES	2	2.501 ± 0.002 b

The small letters, a, b and c, are marked at the 0.05 probability level.

DISCUSSION

Many factors such as medium composition, genotype, explant and their different placements that affected embryogenesis differentiation and plant regeneration have been studied broadly in wheat tissue culture. However, before the ES culturing method for mature embryo tissue culture was explored by Ozgen *et al.* (1998), no desirable results were obtained because of

the low ability of plant regeneration. This method could dramatically increase plant regeneration efficiency up to 96.1%. But in our experiment, the highest plant regeneration efficiency was only 65.8%. The big gap probably arose from the fact that the plantlets that directly grew from mature embryo rather than from calli differentiation were not counted in our experiment. But this was not mentioned in Ozgen's study (1998). To improve regeneration efficiency, the thin mature embryo fragments of wheat were used as explant for plant regeneration (Delporte *et al.* 2001). But only an average of 11% of the embryogenic calli gave rise to plant regeneration; the best protocol produced 25-30 plants per 100 embryos. Moreover, this method used a few excessive steps, such as crushing the embryos into pieces with a metallic pestle, sieving them with a sterile nylon mesh, and characterizing them with image analysis technique, and was hard to manipulate.

Differentiation and regeneration capacity of callus are closely linked to the quality of callus induced. In this study, mature embryos in ES culture had a lower percentage of callus induction than those in NES culture, but induced calli were mainly embryogenic and were further developed into plantlets, resulting in higher culture efficiency. Dedifferentiation of explants is a very complex process that may be involved in the reprogramming of gene expression pattern that changes the fate of the cell. Exposure to high auxin concentrations of the wounded wheat mature embryos caused a change in the normal cell developmental program. During this process, it was possible that some cells in explants in ES culturing might acquire signals from the endosperm, which facilitated the explants' proper differentiation and thus resulted in the production of high-quality plantlets. Another speculation was that the endosperm provided a much better nutrient supply than the artificial medium. Anyway, this interesting fact should be further studied.

Some researchers had indicated that the accumulation of active oxalate oxidase in cell wall could modify the mechanical properties of the wall and consequently prevented its rapid expansion. A substantial callus had developed; the arrested zygotic embryo was completely surrounded by some cells expressing active germin-like oxalate oxidase at a high level, retaining their meristematic and organogenic capacity (Caliskan *et al.* 2004). In this experiment, the regeneration efficiency of calli in ES culture was much higher than that in NES culture. Simultaneously, oxalate oxidase activity was also significantly higher in ES calli than in NES calli. This consistency between high oxalate oxidase activity and high regeneration efficiency was first found in wheat mature embryo culture, and this finding verified that oxalate oxidase might be essential for the formation of embryogenic callus. The higher oxalate oxidase activity in callus might be essential for some cells to acquire embryogenic capacity during dedifferentiation and regeneration. Therefore, the possibility that oxalate oxidase activity could be used as a reliable index to discriminate the embryogenic and non-embryogenic calli was suggested.

The ES culturing significantly improved the regeneration capacity of mature embryos. Though little is known about the molecular mechanisms in relation to the function of endosperm in ES culture, mature wheat embryos can be a valuable explant source for efficient plant regeneration in a wide range of practices such as genetic transformation.

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