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Efficient Callus Induction and Plant Regeneration from Immature and Mature Embryo Culture of Korean Wheat Genotypes

Jung-Hun Moon*, Sang-Kyu Lee*, Kyung-Hee Kim*, Moon-Seok Kang, Hwa-Young Heo**, Young-Up Kwon**, Jung-Hyun Nam**, Yong Weon Seo***, and Byung-Moo Lee*†**

**Department of Plant Resources, Dongguk University, Seoul 100-715, Korea*
***National Crop Experiment Station, RDA, Suwon 441-100, Korea*
****Department of Crop Science, Korea University, Seoul 136-701, Korea*

ABSTRACT: Immature and mature embryos of 18 Korean wheat genotypes were cultured *in vitro* to develop an efficient method of callus formation and plant regeneration, and to compare the responses of both embryo cultures. Immature and mature embryos were placed on a solid agar medium containing the MS salts and vitamins, 30 g/l maltose, 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), and amino acids. The developed calli were maintained on regeneration medium containing MS salts and B5 vitamins, 20 g/l sucrose, and the combination of two plant growth regulators, 6-benzylaminopurine (BA-P) and indole-3-acetic acid (IAA). Immature embryos in most genotypes showed high efficiency of callus induction except three genotypes; Eunpamil, Chunggemil, and Namhaemil, and significant differences among the genotypes. Plant regeneration of calli induced from immature embryos showed high efficiency in Geurumil (56.5%), Tapdongmil (50.5%), Gobunmil (45.5%), and Urimil (42.2%). The analysis of variance showed significant differences for regeneration frequency among the genotypes. Mature embryos showed low callus induction frequency compared with that in immature embryos, and significant differences among the genotypes. Plant regeneration of calli induced from mature embryos showed high efficiency in Keumkangmil (33.33%), Tapdongmil (28.13%), and Geurumil (27.78%). The analysis of variance showed significant differences for plant regeneration frequency among the genotypes.

Keywords : Korean wheat cultivar, immature and mature embryos, callus induction, plant regeneration.

The transformation of all major cereals has now been achieved opening the way for the genetic engineering of new transgenic plants with modified agronomic traits, such as herbicide resistance, biotic and/or abiotic stresses resistance and grain quality and composition (Barro *et al.*, 1999). In general, callus derived from monocots (wheat, barley, maize, etc.) is more difficult to regenerate *in vitro* compared with that from dicots. Wheat is considered the most important field crop and has the largest harvested area and greatest crop production. However, to improve the efficiency of current wheat transformation systems and to enable transformation of cultivars important in wheat production, continuous efforts to improve wheat tissue culture, especially regeneration ability, are needed.

The frequencies of callus induction and plant regeneration in tissue culture of wheat are commonly influenced by genotypes (Fennell *et al.*, 1996; Özgen *et al.*, 1998; Machii *et al.*, 1998; Varshney and Altpeter, 2001), culture media (Fennell *et al.*, 1996; Kang, 1996; Puolimatka and Pauk, 2000) and the explant sources, such as mature and immature embryos (Özgen *et al.*, 1998; Delporte *et al.*, 2001; Özgen *et al.*, 2001), isolated scutellum (Bommineni and Jauhar, 1996; Maees *et al.*, 1996), immature inflorescence (Sharma *et al.*, 1995; Barro *et al.*, 1999), leaf (Jang and Min, 1989), microspores (Hu and Kasha, 1997; Liu *et al.*, 2002), ovule (Kumlehn *et al.*, 1997), and anther (Kang, 1996; Orshinsky and Sadasivaiah, 1997; Zheng and Konzak, 1999; Brisibe *et al.*, 2000).

The best results have been obtained by culturing immature embryos (Rakszegi *et al.*, 2001). Immature embryos are known to be the best explant for efficient regeneration from callus culture of wheat. Though immature embryos have been used frequently as an explant source in wheat tissue culture, it is usually difficult to obtain immature embryos throughout the year, and their acceptable stage for culture is also rigidly limited. Mature embryos which are easily available at all times are not frequently used as explant sources because of their low frequency of callus induction and regeneration. However, the endosperm-supported callus induction method have been used in callus induction from mature embryo cultures (Özgen *et al.*, 1996; Delporte *et al.*, 2001; Özgen *et al.*, 2001).

Although responses of Japanese (Machii *et al.*, 1998) and European (Varshney and Altpeter, 2001) wheat genotypes to tissue culture were reported, Korean wheat genotypes were

†Corresponding author: (Phone) +82-2-2260-3307 (E-mail) bmlee@dongguk.edu
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not well known. Therefore it is important to screen for wheat genotypes with a high ability of callus induction and regeneration. The purpose of this study was to develop an efficient method of callus induction and plant regeneration from mature embryo culture, to compare the responses of mature and immature embryo cultures, and to determine genotypic influences for both cultures in Korean wheat.

MATERIALS AND METHODS

Plant materials

Mature and immature seeds of Korean wheat genotypes were provided by the National Crop Experimental Station in Suwon. Mature seeds of eighteen Korean wheat genotypes were used as target tissues to improve an efficient method of callus induction and regeneration. Immature seeds of sixteen genotypes were used except two genotypes; Dahongmil and Jopummil.

Mature and immature seeds sterilization

Mature seeds were washed several times in soapy water, rinsed in distilled water, surface-sterilized in 70% (v/v) ethanol for 10 min, followed by 50% (v/v) clorox containing three drops of Tween 20 for 1 hour with vigorous shaking, and then rinsed thoroughly with sterile distilled water. Immature seeds were harvested from the main spikes, surface-sterilized in 70% (v/v) ethanol for 1 min, followed by 40% (v/v) clorox containing two drops of Tween 20 for 40 min with vigorous shaking, and then washed several times in sterile distilled water. For callus induction, immature embryos measuring 1.0~2.0 mm in diameter were aseptically removed from surface-sterilized immature seeds.

Callus induction and plant regeneration

Mature embryos and immature embryos were placed on four types media in order to evaluate their ability to initiate calli and to produce embryogenic tissues. Callus induction media were supplemented with the combination of amino acid to investigate the effect of amino acids such as glutamine, proline, asparagine and casein hydrolysate (Table 1). All of callus induction media contained the MS salts and vitamins, 2 mg/l 2,4-D, 30 g/l maltose, and 7 g/l phyto agar. Both mature and immature embryos sources were cultured for 4 weeks at 25°C in the dark and subcultured on the same medium every 2 weeks. Callus induction frequency was calculated as the number of induced calli out of the total number of embryos placed on the callus induction media. After 4 weeks, calli were transferred to the three regeneration media. All of regeneration media contained the MS salts, B5 vitamins, 20 g/l sucrose, and 7 g/l phyto agar, and were supplemented with the combination of two plant growth regulators, BAP and IAA (Table 1). All media of above were adjusted to pH 5.8 and autoclaved for 20 min at 121°C and 1.2 kg/cm^2 pressure. Calli were cultured in regeneration medium at 25°C in 16 h/8 h light/dark provided by a fluorescent light (3000 lux) and subcultured on the same medium every other week. Plant regeneration frequency was calculated as the number of calli showing plant regeneration out of the total number of calli induced.

Statistical analysis

Data were analysed using the SAS (8.01 version) system. The effects of genotype on culture responses were determined by analysis of variance and least significant differences (LSD) tests.

RESULTS AND DISCUSSION

Callus induction

Callus formation from mature and immature embryos was initiated after 3-4 days of culture. On the 4 weeks, the size of

Table 1. Culture media of callus induction and plant regeneration from immature and mature embryos.

Media	Composition
C1	MS salts plus vitamins, 30 g/l maltose and 2 mg/l 2,4-D
C2	MS salts plus vitamins, 30 g/l maltose, 2 mg/l 2,4-D, 500 mg/l Glutamine
C3	MS salts plus vitamins, 30 g/l maltose, 2 mg/l 2,4-D, 500 mg/l Glutamine, 100 mg/l Proline and 100 mg/l Asparagine
C4	MS salts plus vitamins, 30 g/l maltose, 2 mg/l 2,4-D, 500 mg/l Glutamine, 100 mg/l Proline and 100 mg/l Casein hydrolysate
R1	MS salts plus B5 vitamins, 20 g/l sucrose and 0.1 mg/l BAP
R2	MS salts plus B5 vitamins, 20 g/l sucrose, 1 mg/l BAP and 0.5 mg/l IAA
R3	MS salts plus B5 vitamins and 20g/l sucrose

†C : Callus induction media. R : Regeneration media.

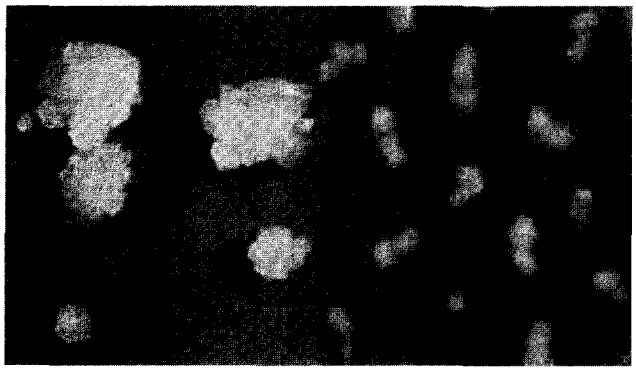

Fig. 1. Callus induced from immature embryos (A) and mature embryos (B) of wheat.

the calli from immature and mature embryos was 6-10mm and 5-8mm, respectively. (Fig. 1). All wheat genotypes produced callus from immature embryos on four media and showed high callus induction frequency except three genotypes; Eunpamil, Chunggemil, and Namhaemil, the callus induction rate ranged from 81% to 100% (Table 2). Machii *et al.* (1998) reported that the callus induction rate from immature embryos of Japanese wheat genotypes ranged from 20% to 100%. The analysis of variance showed significant differences among the genotypes ($P<0.01$) with least significant differences at 4.29% for callus formation, but the differences among four callus induction media across genotypes were not significant. Barro et al. (1999) reported that the highest frequency of callus induction from immature embryos was obtained using an induction medium containing half concentration of amino acids. In mature embryos, calli were produced all wheat genotypes on four media but showed low callus induction frequency compared with immature embryos. (Table 3). The callus induction rate ranged from 36% to 89%. Delporte *et al.* (2001) reported that two or three days later, the callus induction rate from mature embryos reached 100%. The analysis of variance showed significant differences among the genotypes ($P<0.01$) with least significant differences at 13.62% for callus formation, but the differences among the four callus induction media across genotypes were not significant.

Plant regeneration

In both mature and immature embryos, green spots from calli in regeneration media were observed after about 2 weeks (Fig. 2). Multiple shoots were regenerated from calli of immature and mature embryos, and were transferred to test tube for root formation. The analysis of variance showed significant differences among the genotypes ($P<0.01$) with least significant differences at 9.40% for plant regeneration efficiency from immature embryos, but differences among the three regeneration media were not signifi-

Table 2. Percentage of callus formation from immature embryos of 16 Korean wheat genotypes.

Genotype	C1	C2	C3	C4	Mean
Alchanmil	94.82	97.48	97.53	95.01	96.21
Chunggemil	55.03	55.08	58.83	56.27	56.30
Eunpamil	82.53	77.49	81.24	86.23	81.87
Geurumil	98.77	96.25	98.72	100.00	98.43
Gobunmil	98.99	95.96	98.99	98.96	98.22
Jinpummil	95.77	82.85	79.51	93.94	88.02
Jounmil	98.67	100.00	100.00	100.00	99.67
Keumkangmil	100.00	100.00	100.00	100.00	100.00
Milsungmil	100.00	100.00	97.78	100.00	99.44
Namhaemil	84.00	68.67	84.67	79.00	79.08
Olgeurumil	100.00	100.00	100.00	100.00	100.00
Olmil	96.97	94.95	98.99	97.98	97.22
Saeolmil	100.00	93.33	100.00	97.22	97.64
Seodunmil	97.70	98.85	100.00	96.55	98.28
Tapdongmil	100.00	100.00	98.61	98.55	99.29
Urimil	97.28	97.62	98.85	98.92	98.17
Mean	93.78	91.16	93.36	93.66	

†The analysis of variance showed significant differences among the wheat genotypes ($P<0.01$) $LSD_{0.05} = 4.29\%$. Differences among the four media were not significant. Callus induction frequency = number of calli induced/number of immature embryos cultured ×100.

Table 3. Percentage of callus formation from mature embryos of 18 Korean wheat genotypes.

Genotype	C1	C2	C3	C4	Mean
Alchanmil	60.00	70.00	74.92	86.67	72.90
Chunggemil	74.44	91.11	85.56	70.37	80.37
Dahongmil	42.34	44.42	58.49	57.95	50.80
Eunpamil	40.00	30.00	41.59	68.89	45.12
Geurumil	59.44	58.92	65.83	54.92	59.78
Gobunmil	58.33	43.33	54.17	76.67	58.13
Jinpummil	25.56	36.67	20.74	25.93	27.22
Jopummil	59.52	69.05	42.86	61.90	58.33
Jounmil	32.22	55.56	52.94	57.32	49.51
Keumkangmil	77.31	81.67	70.00	76.67	76.41
Milsungmil	27.44	54.09	41.40	56.22	44.79
Namhaemil	55.91	45.73	57.64	50.56	52.46
Olgeurumil	55.44	57.52	66.37	58.52	59.46
Olmil	70.00	56.67	55.83	60.00	60.63
Saeolmil	43.56	55.00	33.33	25.74	39.41
Seodunmil	28.89	38.89	44.44	44.44	39.17
Tapdongmil	86.24	89.20	91.97	90.36	89.44
Urimil	45.56	43.33	29.83	27.33	36.51
Mean	52.35	56.73	54.88	58.36	

†The analysis of variance showed significant differences among the wheat genotypes ($P<0.01$) $LSD_{0.05} = 13.62\%$. Differences among the four media were not significant. Callus induction frequency = number of calli induced/number of mature embryos cultured ×100.

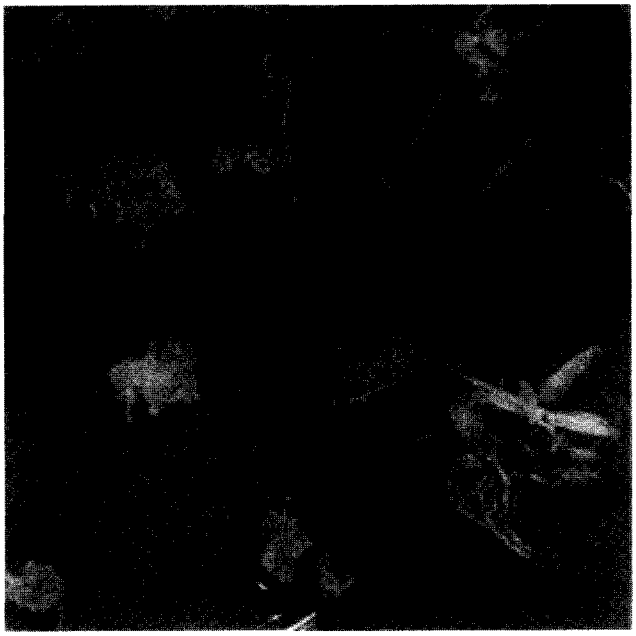

Fig. 2. Plant regeneration from immature embryos (A-B) and mature embryos (C-D) of wheat. Green spot from calli (A, C). Regenerated multiple shoots (B, D).

cant. Plant regeneration efficiency from immature embryos showed high significant in Geurumil (56.55%), Tapdongmil (50.51%), Gobunmil (45.51%), and Urimil (42.23%) (Table 4). In mature embryos, the analysis of variance showed significant differences among the genotypes ($P<0.01$) with least significant differences at 5.63% for plant regeneration efficiency. The three genotypes - Keumkangmil (33.33%), Tapdongmil (28.13%), and Geurumil (27.78%) - were showed high regeneration efficiency compared with other genotypes (Table 5). Genotypic effects on plant regeneration from wheat embryo cultures have been reported previously (Sears and Deckard, 1982; Machii *et al.*, 1998). zgen *et al.* (1998) reported a high genotypic influence on callus induction and plant regeneration from both immature and mature embryos, and suggested that culture responses of mature embryos may be superior to those of immature embryos. The analysis of variance showed no significant differences for regeneration frequency among the three regeneration media. And the callus induction media did not show significant differences for subsequent wheat regeneration. Ahloowalia (1982) reported high regeneration frequency from immature embryos of wheat when calli were induced in media containing IAA and 2,4-D.

In conclusion, there was strong genotypic influence on callus induction and plant regeneration in both mature and immature embryos cultures. The present study showed that frequency of callus induction and plant regeneration from

Table 4. Percentage of plant regeneration from immature embryos of 16 Korean wheat genotypes on three regeneration medium.

Genotype	R1				R2				R3				Mean
	C1	C2	C3	C4	C1	C2	C3	C4	C1	C2	C3	C4	
Alchanmil	69.70	45.45	50.00	37.50	50.00	45.45	38.18	25.00	27.27	38.18	28.64	24.04	39.95
Chunggemil	0.00	0.00	6.25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.52
Eunpamil	23.64	15.00	27.27	26.14	52.27	26.11	40.00	29.17	60.00	15.56	40.91	58.33	34.53
Geurumil	75.00	66.67	33.33	46.43	75.00	50.00	58.33	69.05	30.95	61.90	61.90	50.00	56.55
Gobunmil	50.00	70.83	42.22	47.22	68.33	56.25	27.78	22.22	57.78	31.25	36.67	31.25	45.15
Jinpummil	4.55	25.00	6.25	11.81	0.00	16.67	6.25	19.09	8.71	0.00	0.00	15.00	9.44
Jounmil	14.55	0.00	0.00	0.00	0.00	7.42	10.71	15.00	40.00	3.57	20.00	0.00	9.27
Keumkangmil	16.67	13.39	14.55	13.39	5.56	0.00	0.00	0.00	10.00	7.14	10.00	28.33	9.92
Milsungmil	4.17	7.69	3.85	0.00	5.00	0.00	0.00	6.25	0.00	4.17	0.00	3.57	2.89
Namhaemil	19.09	18.75	0.00	5.56	19.09	0.00	6.25	22.22	23.64	13.64	16.04	0.00	12.02
Olgeurumil	42.31	30.77	25.83	33.24	25.00	32.05	25.83	12.50	28.13	51.92	46.43	29.17	31.93
Olmil	20.00	7.69	10.00	4.55	8.33	0.00	24.04	27.62	14.29	13.26	26.92	22.62	14.94
Saeolmil	12.50	27.78	8.01	8.71	17.42	7.14	17.42	0.00	26.14	18.75	8.33	13.39	13.80
Seodunmil	0.00	3.85	7.14	0.00	8.33	12.50	16.67	0.00	14.84	17.42	18.18	13.64	9.38
Tapdongmil	81.25	68.18	40.00	72.73	50.00	25.00	36.67	28.64	33.04	47.73	57.78	65.15	50.51
Urimil	9.09	48.08	43.56	48.33	32.05	67.95	50.00	46.67	27.78	26.11	57.14	50.00	42.23
Mean	27.66	28.07	19.89	22.23	26.02	21.66	22.38	20.21	21.71	21.72	21.55	21.89	

†The analysis of variance showed significant differences for regeneration among the 16 wheat genotypes ($P<0.01$) $LSD_{0.05} = 9.40\%$. Differences among the media were not significant. Plant regeneration frequency = number of regenerable calli/number of calli induced×100.

Table 5. Percentage of plant regeneration from mature embryos of 18 Korean wheat genotypes on three regeneration medium.

Genotype	R1				R2				R3				Mean
	C1	C2	C3	C4	C1	C2	C3	C4	C1	C2	C3	C4	
Alchanmil	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Chunggemil	10.00	20.00	30.00	0.00	10.00	10.00	10.00	10.00	0.00	10.00	0.00	10.00	10.00
Dahongmil	30.00	10.00	16.67	10.00	30.00	12.50	0.00	10.00	20.00	18.18	16.67	12.50	15.54
Eunpamil	0.00	10.00	0.00	0.00	0.00	0.00	10.00	10.00	0.00	0.00	0.00	10.00	3.33
Geurumil	20.00	33.33	30.00	33.33	20.00	30.00	33.33	33.33	20.00	40.00	20.00	20.00	27.78
Gobunmil	30.00	33.33	10.00	10.00	20.00	0.00	20.00	20.00	0.00	14.29	11.11	20.00	15.73
Jinpummil	20.00	20.00	20.00	10.00	20.00	0.00	0.00	20.00	10.00	0.00	0.00	10.00	10.83
Jopummil	25.00	21.05	18.18	22.22	22.22	15.38	0.00	22.22	22.22	23.08	14.29	22.22	19.01
Jounmil	15.00	23.33	13.64	23.33	29.63	23.33	13.33	25.00	30.00	20.00	13.33	16.67	20.55
Keumkangmil	20.00	30.00	40.00	30.00	30.00	40.00	30.00	30.00	30.00	40.00	30.00	50.00	33.33
Milsungmil	10.87	13.64	17.39	16.67	33.33	11.54	14.71	20.00	16.67	20.00	10.53	18.60	16.99
Namhaemil	20.00	10.00	10.00	10.00	0.00	10.00	10.00	0.00	10.00	11.11	10.53	11.76	9.45
Olgeurumil	13.33	23.33	33.33	20.00	23.33	13.33	20.00	30.00	15.00	20.00	25.00	30.00	22.22
Olmil	10.00	0.00	0.00	10.00	10.00	10.00	15.00	15.00	10.00	0.00	10.00	15.00	8.75
Saeolmil	20.00	20.00	0.00	10.00	0.00	10.00	0.00	0.00	0.00	10.00	0.00	20.00	7.50
Seodunmil	0.00	0.00	0.00	0.00	16.67	14.29	0.00	0.00	0.00	0.00	0.00	0.00	2.58
Tapdongmil	27.50	35.71	27.50	33.33	10.71	27.78	30.00	30.00	25.00	36.67	26.67	26.67	28.13
Urimil	15.00	0.00	10.00	15.00	0.00	20.00	10.00	10.00	10.00	10.00	10.00	0.00	9.17
Mean	16.48	17.43	15.93	14.66	15.88	14.34	12.58	16.42	12.72	15.74	11.56	16.68	

†The analysis of variance showed significant differences for regeneration among the 18 wheat genotypes ($P<0.01$) $LSD_{0.05}=5.63\%$. Differences among the media were not significant. Plant regeneration frequency = number of regenerable calli/number of calli induced×100.

mature embryos were low compared with immature embryos. However, genotypic effects had a similar tendency in callus induction and plant regeneration of both explant types. Therefore, mature embryos which are readily available throughout the year, can be used as an effective explant source in wheat tissue culture.

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