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Improvement of Regeneration Efficiency from Mature Embryo and Leaf Base Segment in Korean Oat Genotypes

Kyung-Hee Kim, Jung-Hun Moon, Sang-Kyu Lee, and Byung-Moo Lee†

Department of Plant Resources, Dongguk University, Seoul 100-715, Korea

ABSTRACT: Mature embryo and leaf base segment of Korean oat were used as materials in an experiment to check plant regeneration efficiency. MS media supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin, and picloram were used for callus induction from mature embryos and leaf base segments. Three mg/l of 2,4-D and 3 mg/l of picloram in callus induction medium showed high frequency for plant regeneration from mature embryos. Leaf base segments were transferred to callus induction medium and incubated at 25 °C in 16/8 hr light/dark cycle for 3 weeks. Callus induction from leaf base segments of Malgwiri showed high efficiency in medium containing 3 mg/l of 2,4-D and 1 mg/l of kinetin (91.8%). In case of Samhangwiri, the combinations of phytohormones did not show significant difference. Regeneration from leaf base segments showed high frequency in shoot medium containing 1 mg/l of antiauxin, tri-iodobenzoic acid (TIBA) and 1 mg/l of 6-benzyladenine (BA). Calli induced from leaf base segments of Samhangwiri and Malgwiri in media containing 3 mg/l of 2,4-D and 3 mg/l of picloram showed high regeneration frequency. It appears that the callus initiation medium may be an important factor for subsequent plant regeneration.

Keywords: Korean oat, mature embryo, leaf base segment, callus induction, plant regeneration

The most frequently and successfully used explants in oat tissue cultures are immature embryos (Bregitzer *et al.*, 1991; Cummings *et al*, 1976; Lorz *et al*, 1976; Rines & Luke, 1985; Rines & McCoy, 1981) and mature embryos (Birsin *et al.*, 2001, Cater *et al.*, 1967; Cummings *et al.*, 1976; Heyser & Nabors, 1982, Kim & Lee, 2002). However, harvest of immature embryos are only available at a very limited time in a growing season and generally requires growth of oat plants in climate-controlled room (Tobert *et al.*, 1998a). Mature embryos are readily available at any time period. Therefore, mature embryos can be used as an effective and convenient explant source in plant transformation, even thought its efficiency is relatively lower than immature seed. Among the explants used for the induction of regenerable cultures, young seedlings are the most easily available donor materials. Since they can be grown in a short term, incessant supply of explants can be provided (Jahne-Gartner & H. Lörz, 1996). An efficient *in vitro* regeneration system for oat using leaf bases as explants has been developed (Chen *et al.*, 1995; Chen Z *et al.*, 1995; Gless *et al.*, 1998a; Gless *et al.*, 1998c; Nuutila *et al.*, 2002; Zhang *et al.*, 1996). The leaf base segments as a target tissue in gene transfer experiments has used because of the short cultivation period required *in vitro*, which decreases the hazard of variation (Gless *et al.*, 1998b). Embryogenic cultures from immature and mature embryos (Somers *et al.*, 1992; Tobert *et al*, 1998a) and from leaf-base segments of oat (Gless *et al*, 1998b) have also been used as a target for gene transfer by particle bombardment.

The combination of a high concentration of 2,4-D with a low level of kinetin in the callus induction medium was essential in order to obtain plant regeneration from induced embryo formation (Kiviharju & Taurialnen, 1999). Besides, Kim & Lee (2002) reported that high concentration of 2,4-D and low concentration of kinetin in mature embryos of oat were beneficial for plant regeneration. Antiauxin, TIBA, was reported for its beneficial effect in maize anther culture (Büter, 1997). It is known that TIBA reduced the restraint action of any residual 2,4-D and also had a positive influence on the regeneration potential of the calli. Considering all that facts reported, it is easy to draw a conclusion that auxins such as 2,4-D play a key role in somatic embryo induction. However, its continuous presence retards further progression of embryo specialization and growth.

The aims of this work were to examine the effect of plant growth regulators on oat regeneration from mature embryo and leaf base segment and to develop an efficient method for gene transfer in Korean oat genotypes.

MATERIALS AND METHODS

Preparation and culture of mature embryos and leaf base segments

Mature seeds of Malgwiri and Samhangwiri were sterilized for 5 min in 70% ethanol. And then seeds were steril-

†Corresponding author (Phone) +82-2-2260-3307 (E-mail) bmlee@dongguk.edu
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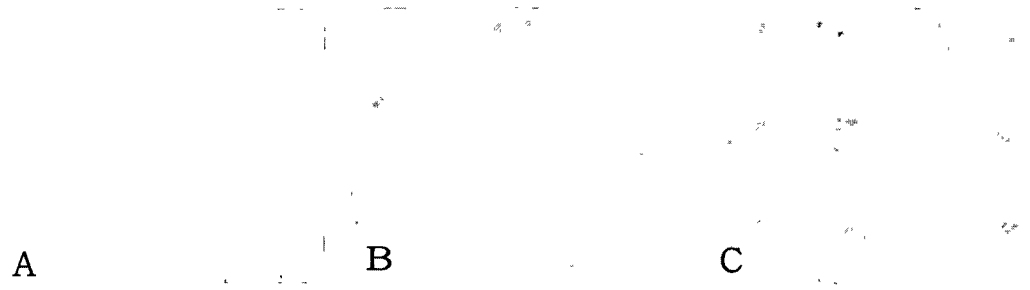

Fig. 1. Regeneration from leaf base segments A: Five-day-old seedling, B. Three-week-old basal segment calli, C Shoot regeneration from leaf base on MS medium containing 1 mg/l of TIBA and 1 mg/l of BA.

ized with 60% Clorox® containing 3 drops of Tween 20 per 100 ml for 45 min with gentle shaking and rinsed three times with sterile distilled water and placed on media for callus induction in the dark at 25 °C

Seedlings from mature seeds were cultured in the light at 25 °C on liquid MS medium (Fig. 1A). Seedlings with a leaf length about 5 cm (from 4-day-old) were cut 1 to 2 mm segments. However, the shoot with leaf base segments was confined within the first 1 to 2 mm above the region of root initiation.

Callus induction

The medium were supplemented with the combinations of 2,4-D, kinetin, and picloram to evaluate the effect of phytohormones on callus induction from mature embryos and leaf base segments (Table 1). Mature embryos were cultured on MS medium (Murashige & Skoog, 1962) in the dark at 25 °C for 4 weeks. Leaf base segments were transferred to callus induction medium and incubated at 25 °C in 16 hr light and 8 hr dark condition for 3 weeks.

Table 1. Hormones combinations of the callus induction media for mature embryo and leaf base segment.

Callus medium	Hormone composition
C1	3 mg/l 2,4-D
C2	3 mg/l 2,4-D, 1 mg/l kinetin
C3	3 mg/l 2,4-D, 3 mg/l picloram

Plant regeneration

Calli from mature embryos were cultured on regeneration medium at 25 °C in the light condition. MS media were supplemented with the combination of 0.2 mg/l of naphthaleneacetic acid (NAA) and 1 mg/l of BA. After leaf segments were cultured for 3 weeks on callus induction medium, the calli from leaf base segments were transferred to regeneration media. For regeneration, MS media were supplemented with the combinations of TIBA, BA, and kinetin (Table 2) Calli were incubated at 25 °C in 16 hr light and 8 hr dark period.

Table 2. Plant growth regulator compositions in MS media for regeneration from mature embryo and leaf base segment

Explants	Media composition
Leaf base segments	1 mg/l TIBA, 1 mg/l BA 1 mg/l TIBA, 1 mg/l kinetin
Mature embryos	0.2 mg/l NAA, 1 mg/l BA

RESULTS AND DISCUSSION

Effect of different phytohormones on callus induction and plant regeneration from mature embryo of oat

Callus induction from mature embryos of Malgwiri (97.5 %) and Samahangwiri (98.3%) showed high efficiency in medium containing 3 mg/l of 2,4-D and 3 mg/l of picloram (Table 3). Similar result was reported by Kim & Lee (2002). They reported that 3 mg/l of 2,4-D in the medium showed the high callus induction rates in Malgwiri (82.5%) and Samhangwiri (90%) from mature embryo.

Plant regeneration efficiency was examined in MS medium containing 0.2 mg/l of NAA and 1 mg/l of BA. Percentage of plant regeneration from mature embryos of Malgwiri and Samhangwiri showed high in the calli induced from medium containing 3 mg/l of 2,4-D and 3 mg/l of picloram (Table 3). However, Kim & Lee (2002) reported that treatment with 3 mg/l of 2,4-D and 1 mg/l of kinetin in callus medium showed high frequency for plant regeneration in Malgwiri and Samhangwiri Kiviharju & Taurianen (1999) reported that high 2,4-D and low kinetin in anther culture of oat were beneficial for plant regeneration. This result supports that plant regeneration was influenced by the callus initiation medium (Kim & Lee, 2002). The regeneration efficiencies of Malgwiri and Samhangwiri were 74% and 70.8% in media containing picloram and 2,4-D Piclo-

Table 3. Percentage of callus induction and regeneration from mature embryos of oat

	Callus induction		Regeneration (0.2 mg/l NAA, 1 mg/l BA)	
	Malgwiri	Samhangwiri	Malgwiri	Samhangwiri
C1†	93 8	97 4	53.5	61.8
C2	83 4	76.8	61.8	65.4
C3	97.5	98.3	74.0	70.8

†Callus media. C1: MS + 3 mg/l 2,4-D, C2. MS + 3 mg/l 2,4-D, 1 mg/l kinetin, C3: MS + 3 mg/l 2,4-D, 3 mg/l picloram

ram and 2,4-D based media increased significantly the frequencies of callus induction and regeneration (Wernike & Milkovits, 1987). This result may be partly explained by the fact that picloram based media induced higher regeneration frequencies than media containing 2,4-D (Barro *et al.*, 1998). Barro *et al.* (1998) also reported that the highest frequencies of transgenic plant production were 3.2% for inflorescence cultures and 2.5% for scutellum cultures, in both cases from cultures induced on media containing 4 mg/l of picloram. The mechanism of action of picloram was not be determined (Barro *et al.*, 1998). However, it is known that auxins play a role in the activation of genes involved in cell dedifferentiation and division (Dudits *et al.*, 1991) and that cells in the S-phase (DNA synthesis phase) of the cell cycle are more predisposed to the integration of foreign DNA (Villemont *et al.*, 1997).

Effect of hormone combinations on plant regeneration system from leaf base segments

Callus induction rates of Malgwiri from leaf base segments showed high efficiency in medium containing 3 mg/l of 2,4-D and 1 mg/l of kinetin (91.8%) (Table 4). But, in case of Samhangwiri, the combinations of phytohormones did not show difference. Calli from leaf base segments were induced within 1 week. In the beginning, the calli appeared to be creamish, soft, and quickly developed. After 3 weeks of culture, calli showed shoot formation in Malgwiri and Samhangwiri (Fig. 1B). Multiple shoots were produced in regeneration media containing 1mg/l of TIBA and 1 mg/l of BA, and 1 mg/l of TIBA and 1 mg/l of kinetin in Samhangwiri and Malgwiri (Fig. 2). Multiple spots of calli rapidly developed shoots and leaves in regeneration media (Fig. 1C).

Highest regeneration frequencies of Samhangwiri and Malgwiri were obtained with callus media containing 3 mg/l of 2,4-D and 3 mg/l of picloram Whereas, 3 mg/l of 2,4-D did not affect the efficiency of regeneration. After 3 weeks of culture, shoot initiation period from calli decreased in regeneration media containing 1 mg/l of TIBA and 1 mg/l of BA, and 1 mg/l of TIBA and 1 mg/l of kinetin. Best regeneration results were obtained from the media containing 1 mg/l of TIBA and 1 mg/l of BA. Antiauxin TIBA were found to

Table 4. Percentage of callus induction from the leaf base segment of oat

Genotype	C1†	C2	C3
Malgwiri	66	91.8	84.3
Samhangwiri	65 7	63 4	67 8

†Callus media C1: MS + 3 mg/l 2,4-D, C2 MS + 3 mg/l 2,4-D, 1 mg/l kinetin, C3: MS + 3 mg/l 2,4-D, 3 mg/l picloram

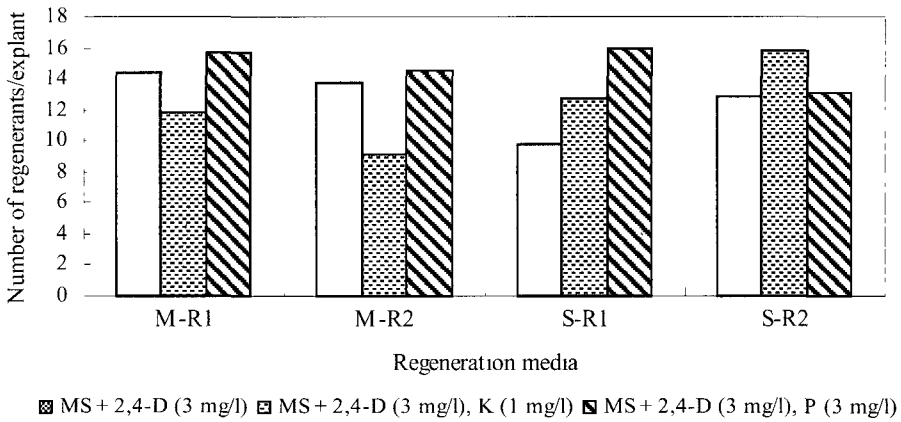

Fig. 2. Influence of phytohormone on the regeneration frequency from leaf base segments of oat
M: Malgwiri, S Samhangwiri R1 MS + 1 mg/l TIBA, 1 mg/l BA, R2· MS + 1 mg/l TIBA, 1 mg/l kinetin

Table 5. Percentage of regeneration from the leaf base segment of oat.

Genotype	MS+TIBA+BA†			MS+TIBA+kinetin		
	MS1‡	MS2	MS3	MS1	MS2	MS3
Malgwiri	65	56.2	69 2	57 1	45 4	64.5
Samhangwiri	54.5	80	81 8	52.3	75	68 1

†Regeneration media : MS + 1 mg/l TIBA, 1 mg/l BA, MS + 1mg/l TIBA, 1 mg/l kinetin Calli were cultured for 3 weeks.
‡MS1 3 mg/l 2,4-D, MS2 3 mg/l 2,4-D + 1 mg/l kinetin, MS3 3 mg/l 2,4-D + 3 mg/l picloram.

be beneficial in maize anther culture (Büter, 1997). TIBA is a known auxin polar transport inhibitor, which in many instances can have negative influence on the development and structural aspects of somatic embryo mutration (Liu *et al.*, 1993, Choi *et al.*, 1997). Chugh & Khurana (2003) reported that high regeneration efficiency was obtained on a regeneration medium containing ethylene action inhibitor, 10 mg/l of silver nitrate and 1 mg/l of TIBA along with 0.4 mg/l of kinetin. The regeneration efficiencies of *T. aestivum* and *T dicoccum* were 68.0% and 80 3% in media containing silver nitrate, TIBA, and kinetin. This appears that antiauxin, TIBA, was found which had a positive influence on the regenerating potential of the calli. The time necessary for the preparation of the target tissue is short and the risk of somaclonal variation is reduced as the period in culture is reduced to a few weeks (Jähne *et al.*, 1995).

In conclusion, frequency of plant regeneration from mature embryos showed high in medium containing 3 mg/l of 2,4-D and 3 mg/l of picloram. Picloram in combination with 2,4-D induced significantly more explants to proceed to wards regeneration : the regeneration efficiency was 74% for picloram based medium of Malgwiri. Multiple shoot frequency was increased with regeneration media containing 1 mg/l of TIBA and 1 mg/l of BA. Besides, shoot initiation period was decreased. The TIBA in combination with BA increased shoot initiation in leaf base segments of oats. Accordingly, these experiments were developed a simple and short-term *in vitro* regeneration system and improved regeneration efficiency

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