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*Full Length Research Paper*

# Factors influencing callus induction and plant regeneration of Dahurian wildrye grass (*Elymus dahuricus* L.)

**Ki-Won Lee#, Ochirbat Chinzorig#, Gi Jun Choi, Ki-Yong Kim, Hee Chung Ji, Hyung Soo Park, Won Hoo Kim and Sang-Hoon Lee***

Grassland and Forages Division, National Institute of Animal Science, Rural Development Administration, Cheonan, 330-801, Korea.

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**Establishment of highly efficient and reproducible regeneration system would greatly influence the efforts of improvement of *Elymus* sp. through gene transfer technology. A suitable callus induction and efficient regeneration protocol for** Dahurian wildrye grass **(Elymus dahuricus L.) was developed. It consisted of 3.0 mg/L 2,4 dichlorophenoxyacetic acid (2,4-D) or Dicamba + 1.0 g/L** casein **hydrolysate (CH) + 300 mg/L L-proline + 30 g/L sucrose in MS, and showed the highest percentage of callus induction. Highest (51%) regeneration was obtained from N6 medium containing 1.0 mg/L 2,4-D + 3.0 mg/L $N_6$-benzylaminopurine (BA) + 1.0 g/L CH + 300 mg/ L L-proline + 30 g/L sucrose. Thus, optimization of regeneration frequency using mature seeds as explants material may offer a simple and efficient protocol for** Dahurian wildrye grass **that may improve molecular breeding of this species.**

**Key words:** Dahurian wildrye grass, Elymus dahuricus L., callus, plant regeneration.

## INTRODUCTION

Dahurian wildrye grass (Elymus dahuricus L.) is a short-lived perennial forage commonly planted in the grassland area of Mongolia. Forage crops are constantly subjected to primary multiple climatic stresses such as high or low temperatures and deficient or excess moisture, soil constraints, and biotic stresses. These environmental stresses are the major limiting factor in forage productivity (Duncan and Carrow, 2001). Recently, molecular breeding such as genetic transformation has become a popular biotechnological tool for improving forage quality as well as improving tolerance to various environmental stresses (Spangenberg et al., 1998). Transgenic technologies developed for genetic manipulation of forage grasses have opened up opportunities for forage improvement (Wu et al., 2005; Hu et al., 2005; Lee et al., 2007). The biggest challenge however, is how to apply the technology to generate novel genetic variability in a way that satisfies regulatory requirements and then efficiently incorporate the new germplasm into breeding programs for cultivar development. Therefore, use of biotechnological tools including *in vitro* culture or genetic transformation may be considered for the improvement of quality and enhancement of resistance to different abiotic or biotic stresses of forage.

Nowadays, mature seeds are considered an important source of explants, and are extensively used for plant regeneration via callus culture (Lee et al., 2006, 2008). Therefore, we made an attempt to develop an efficient regeneration system from mature seed-derived callus of Dahurian wildrye grass for genetic transformation. In order to optimize the callus induction and plant regeneration from seed-derived callus, a broad spectrum of cultural conditions was examined. Establishment of efficient and highly reproducible regeneration system would greatly influence the efforts of improvement of *Elymus* grass species through useful genes transfer technology.

*Corresponding author. E-mail: sanghoon@korea.kr. Tel: +82-41+580-6754.

# Both authors contributed equally to this work

## MATERIALS AND METHODS

Mature seeds of Dahurian Wildrye Grass (*E. dahuricus* L.) local ecotype were supplied by the Mongolian Forage Seed Producers Association. These seeds were dehusked using in 50% sulfuric acid ($H_2SO_4$) and rinsed with distilled water five times then 70% ethanol for 1 min. After rinsing with distilled water three times, seeds were surface-sterilized by immersion in a solution of 30% (w/v) sodium hypochlorite for 30 min, and then two drops of Tween-20 were added. To remove the surfactants, sterilized seeds were rinsed five times with sterile deionized-distilled water and blotted onto a sterile Whatman filter paper.

### Embryogenic callus induction

Sterilized dry seeds were placed on MS basal medium (Murashige and Skoog, 1962) containing 30 g/L sucrose, 1.0 g/L casein hydrolysate (CH), 300 mg/L L-proline and 3 g/L Gelite. About 25 seeds were transferred on each plate (87 × 15 mm) containing callus induction medium. The cultures were transferred to controlled growth chamber at 24 ± 2°C under continuous dim light condition. After four weeks, callus was removed manually from the germinating shoots and was then sub-cultured on the same medium.

### Plant regeneration

After six weeks of culture, embryogenic callus were placed on N6 (Chu et al., 1975) basal medium containing 30 g/L sucrose, 1 g/L CH, 300 mg/L L-proline and 3g/L Gelite, and kept under fluorescent light (80 µE $m^{-2} s^{-1}$) at 16 h photoperiod. For all experiments, pH was adjusted to 5.7 before autoclaving. After four weeks, regenerated shoots (1 to 2 cm) were sub-cultured on the same medium for further development.

### Rooting and acclimation of regenerated plants

For the development of a strong root system, regenerated shoots were further transferred to the rooting medium consisting 1/2 MS with 15 g/L sucrose. Cultures for rooting were kept under fluorescent light (80 µE $m^{-2} s^{-1}$) at 24 ± 2°C and 16 h photoperiod for a couple of weeks. Plantlets with well developed roots were removed carefully from the agar gel and transferred to the plastic pots.

### Factors evaluated

To optimize suitable growth regulators and their optimum concentration and combinations for seed-derived callus induction, different kinds of auxins [2,4-D, Dicamba and α-naphthaleneacetic acid (NAA)] alone or in combination with BA was used. Types and levels of growth regulators were recorded. To elucidate the appropriate condition for plant regeneration, two auxins (2,4-D and NAA) and two cytokinins (BA and kinetin) combinations at various concentrations were used. To determine the role of basal medium for callus induction and plant regeneration, a wide range of saccharides: glucose, sucrose, maltose and mannitol at a concentration (30 g/L) were tested with an optimum level of growth regulator selected basal media. Furthermore, three different kinds of basal medium such as MS, N6 and SH (Schenk and Hildebrandt, 1972) were investigated to determine the effect of each basal medium on callus induction and plant regeneration.

## RESULTS AND DISCUSSION

### Effect of growth regulators to callus induction

Callus induction frequency was measured as percentage of seeds that produced callus. To examine the effect of growth regulators on callus induction, first, various kinds of auxins (2,4-D, Dicamba and NAA) were used alone at different concentrations (1.0 to 10 mg/L). The callus induction frequency varied from 58.3 to 19.7%. Among all auxins, both 2,4-D and Dicamba at a concentration of 3.0 mg/L each produced 56.9 and 58.3% callus, respectively (Table 1). Also, callus weight and morphogenic responses varied according to growth regulators and their concentrations. Dicamba showed slightly higher weight of callus than 2,4-D. Effect of 2,4-D and Dicamba on callus induction frequency was not significant. Among all auxins, 2,4-D has been widely used for callus induction for the plants of the Gramineae family (Sirkka and Immonen, 1993; Bai and Qu, 2000; Chaudhury and Qu, 2000). In our studies, callus induction frequency was examined by single use of different kinds of auxins at various concentrations. Single use of 2,4-D or 3 mg/L Dicamba in MS media was found to increase the callus induction frequency. Therefore, we used 2,4-D because of its low cost than Dicamba.

### Effect of growth regulators on plant regeneration

To examine the effect of plant growth regulators on plant regeneration, auxins (2,4-D and NAA) and cytokinins (BA and kinetin) combinations were used at various concentrations. Green shoots were observed within two to three weeks of culture (Figure 1C and D). Among all concentrations and combinations, 1.0 mg/L of 2,4-D and 3.0 mg/L of BA showed the highest percentage (51.1%) of shoot regeneration as well as early shoot induction (Table 2). Thus, combination of the optimum levels of auxin with a high level of cytokinin for plant regeneration showed better results, which were statistically significant compared to the effects of a low level of cytokinin. Moreover, combination of the low levels of auxin with optimum levels cytokinin for plant regeneration showed far better results, which were statistically significant compared to the effects with a high level of cytokinin.

### Effect of carbon sources to callus induction and regeneration

Various types of carbon sources such as monosaccharides (glucose, mannitol) and disaccharides (sucrose and maltose) were examined to evaluate their respective effects on callus induction and subsequent plant regeneration. Significant differences were found among carbon sources tested for callus formation and subsequent regeneration. Disaccharides performed

**Table 1.** Effect of different concentrations of auxins on callus formation from mature seeds of Dahurian Wildrye Grass (*Elymus dahuricus* L.).

| Auxins | Concentration (mg/L) | Number of seeds tested | Number of callus induced[a] | Callus formation (%) | Callus fresh weight (mg)[b] |
|---|---|---|---|---|---|
| 2,4-D | 0 | 60 | 0 | 0 | 0 |
| | 1 | 120 | 32 | 26.7 ± 1.4 | 21 ± 3.6 |
| | 3 | 120 | 68 | 56.9 ± 2.4 | 49 ± 2.6 |
| | 5 | 120 | 57 | 47.8 ± 1.3 | 46 ± 3.1 |
| | 7 | 120 | 50 | 41.4 ± 1.7 | 41 ± 2.5 |
| | 10 | 120 | 27 | 22.2 ± 2.1 | 31 ± 1.0 |
| Dicamba | 0 | 60 | 0 | 0 | 0 |
| | 1 | 120 | 33 | 27.8 ± 1.0 | 30 ± 1.5 |
| | 3 | 120 | 70 | 58.3 ± 2.2 | 48 ± 3.1 |
| | 5 | 120 | 58 | 48.3 ± 1.7 | 50 ± 1.5 |
| | 7 | 120 | 44 | 36.4 ± 2.4 | 44 ± 1.5 |
| | 10 | 120 | 28 | 23.6 ± 0.5 | 40 ± 2.0 |
| NAA | 0 | 60 | 0 | 0 | 0 |
| | 1 | 120 | 25 | 21.1 ± 2.1 | 18 ± 2.1 |
| | 3 | 120 | 50 | 41.9 ± 1.3 | 42 ± 2.0 |
| | 5 | 120 | 36 | 30.3 ± 1.7 | 41 ± 2.6 |
| | 7 | 120 | 39 | 32.8 ± 1.3 | 40 ± 4.0 |
| | 10 | 120 | 24 | 19.7 ± 1.0 | 34 ± 2.0 |

[a]Mature seeds were cultured on MS medium containing 0 to 10 mg/L auxins, 30 g/L sucrose, 1.0 g/ L CH, 300 mg/L L-proline, 3 g/L gelrite, and cultured for four weeks. [b]Values represent mean of callus fresh weight formed from one seed.

**Table 2.** Effect of growth regulators on plant regeneration of Dahurian wildrye grass (*Elymus dahuricus* L.).

| Growth regulator (mg/L) | | Plant regeneration (%)[a] |
|---|---|---|
| 2,4-D | BA | |
| 1 | 1 | 30.0 ± 1.0 |
| | 3 | 51.1 ± 1.5 |
| | 5 | 38.9 ± 3.1 |
| 2 | 1 | 26.7 ± 2.0 |
| | 3 | 31.1 ± 1.5 |
| | 5 | 26.7 ± 1.0 |
| 2,4-D | kinetin | |
| 1 | 1 | 24.4 ± 2.1 |
| | 3 | 36.7 ± 1.0 |
| | 5 | 38.9 ± 2.5 |
| 2 | 1 | 22.2 ± 2.5 |
| | 3 | 21.1 ± 1.5 |
| | 5 | 13.3 ± 1.0 |
| NAA | BA | |
| 1 | 1 | 28.9 ± 1.5 |
| | 3 | 42.2 ± 1.5 |
| | 5 | 38.9 ± 1.2 |

**Table 2.** Continue.

| | | |
|---|---|---|
| | 1 | 17.8 ± 1.5 |
| 2 | 3 | 18.9 ± 1.5 |
| | 5 | 21.1 ± 1.7 |
| | | |
| NAA | kinetin | |
| | 1 | 35.6 ± 2.1 |
| 1 | 3 | 40.0 ± 2.0 |
| | 5 | 25.6 ± 1.5 |
| | | |
| | 1 | 14.4 ± 2.1 |
| 2 | 3 | 16.7 ± 2.6 |
| | 5 | 11.1 ± 1.5 |

[a]Calli were transferred to N6 medium containing 30 g/L sucrose, 1.0 g/L CH, 300 mg/L L-proline 3 g/L gelite, and cultured for six weeks.

**Table 3.** Effect of carbon sources on callus formation and plant regeneration in mature seeds cultures of Dahurian wildrye grass (*Elymus dahuricus* L.).

| Carbon source | Number of seeds transferred[a] | Callus formation (%) | Number of calli transferred | Plant regeneration (%)[b] |
|---|---|---|---|---|
| Sucrose | 120 | 58.3 ± 1.5 | 100 | 55 ± 1.7 |
| Maltose | 120 | 49.2 ± 0.6 | 100 | 50 ± 2.1 |
| Glucose | 120 | 36.7 ± 2.1 | 100 | 44 ± 1.5 |
| mannitol | 120 | 20.8 ± 1.5 | 100 | 19 ± 2.1 |

[a]Mature seeds were cultured on MS medium containing 0 to 10 mg/L auxins, 30 g/L sucrose, 1.0 g/L CH, 300 mg/L L-proline, 3 g/L gelrite and cultured for 4 weeks. [b]Calli were transferred to N6 medium containing 30 g/L sucrose, 1.0 g/L CH, 300 mg/L L-proline, 3 g/L gelite, and cultured for 6 weeks.

**Table 4.** Effect of basal media on callus formation and plant regeneration in mature seeds cultures of Dahurian wildrye grass (*Elymus dahuricus* L.).

| Media | Number of seeds transferred | Callus formation (%)[a] | Number of calli transferred | Plant regeneration (%)[b] |
|---|---|---|---|---|
| MS | 120 | 56.7 ± 2.1 | 100 | 52 ± 2.5 |
| N6 | 120 | 45.0 ± 1.0 | 100 | 57 ± 2.0 |
| SH | 120 | 29.2 ± 2.1 | 100 | 29 ± 1.5 |

[a]Mature seeds were cultured on MS medium containing 0 to 10 mg/L auxins, 30 g/L sucrose, 1.0 g/L CH, 300 mg/L L-proline, 3 g/L gelrite and cultured for 4 weeks. [b]Calli were transferred to N6 medium containing 30 g/L sucrose, 1.0 g/L CH, 300 mg/L L-proline, 3 g/L gelite, and cultured for 6 weeks.

better than monosaccharides. The highest (58.3%) mature seeds induced callus and 55% shoot regeneration was observed when 30 g/L of sucrose was added as a carbon source (Table 3). Carbon source is one of the key factors in *in vitro* tissue culture systems. Fuentes et al. (2000) reported that specificity of carbohydrates may have differential effects on morphogenesis. Even though it is well documented that sucrose is the best source of carbon for *in vitro* systems, there are also some reports where maltose was found to give better results than other carbohydrates (Ainsley and Aryan, 1998; Chang et al., 2003; Sharma et al., 2005).

## Effect of basal medium on callus induction and plant regeneration

Three different kinds of basal mediums (MS, N6 and SH) were used to investigate their effects on callus induction and regeneration. Significant differences in callus induction and plant regeneration frequency were observed (Table 4). The highest (56.7%) seeds callused

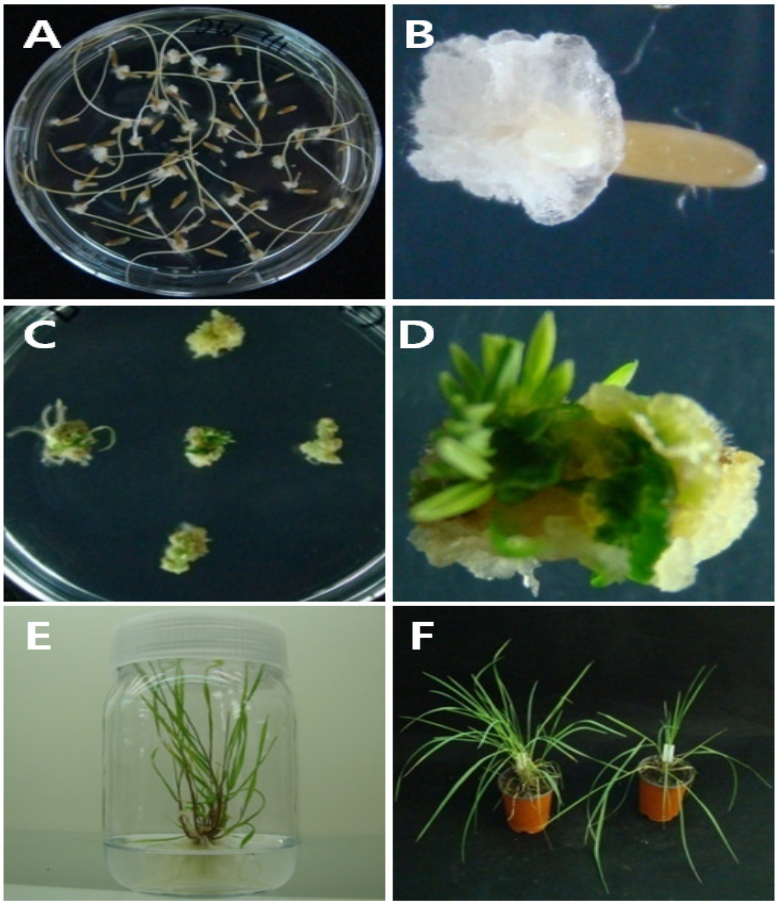


**Figure 1.** Plant regeneration from mature seed-derived callus of Dahurian wildrye grass. (A, B) Calluses appeared from the mature seeds; (C, D) regeneration of shoots from embryogenic calli in the regeneration medium; (E) regenerated plants in the rooting medium; (F) plantlets growing in the pot.

when they were cultured on MS medium under previously described optimized conditions of growth regulators and carbon source. Whereas N6 basal medium appeared to the best medium for maximum (57%) shoot regeneration, in SH medium, both volume of callusing and plant regeneration frequency were lower compared to other media. It is reported that the basal medium plays a significant role in callus induction and somatic embryogenesis (Ainsley and Aryan, 1998; Zhao et al., 1999; Lee et al., 2002).

In order to optimize the callus induction and the subsequent regeneration from mature seed-derived callus, a broad spectrum of cultural conditions was examined and appropriate conditions were selected through stepwise optimization. Through this experiment, we established tissue culture systems that will enable efficient attainment of regenerated plants from Dahurian wildrye grass mature seeds (Figure 1). Establishment of highly efficient and reproducible regeneration system would greatly influence the efforts of improvement of Dahurian wildrye grass through gene transfer technology.

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