The Journal of Animal & Plant Sciences, 22(2): 2012, Page: 518-521
ISSN: 1018-7081

CALLUS INDUCTION AND PLANT REGENERATION FROM MATURE SEEDS OF SIBERIAN WILDRYE GRASS (*ELYMUS SIBIRICUS* L.)

K. W. Lee†, O. Chinzorig†, G. J. Choi, K. Y. Kim, H. C. Ji, H. S. Park, and S. H. Lee*

Grassland and Forages Division, National Institute of Animal Science, Rural Development Administration, Cheonan, 330-801, Korea.
Corresponding Author e-mail: sanghoon@korea.kr

ABSTRACT

In order to optimize tissue culture conditions of Siberian wildrye grass, the effects of plant growth regulators and various carbon sources on callus induction and plant regeneration was investigated with mature seeds. The optimal concentration of 2,4-D for the induction of primary callus from mature seeds was 5 mg/L. The highest embryogenic callus frequency was observed when the mature seed were cultured on MS medium supplemented with 5 mg/L 2,4-D and 0.1 mg/L BA(6-benzyladenine). The highest plant regeneration frequency was observed when callus was transferred to N6 medium supplemented with 1 mg/L 2,4-D and 3 mg/L BA. Regenerated plants were rooted at the highest rate (100%) when transferred onto 1/2 MS medium. Regenerated plants were morphologically uniform with normal growth pattern. A short tissue culture period and regeneration system would be beneficial for molecular breeding of Siberian wildrye grass by the production of transgenic plant.

Key words: Siberian wildrye grass, *Elymus Sibiricus* L., Callus, Plant regeneration, Grassland.

INTRODUCTION

Siberian wildrye grass (*Elymus Sibiricus* L.) is a species of perennial gramineous forage that is widely distributed ranging from East Europe to Asia and North America (Wang *et al*., 2009). In particular, Siberian wildrye grass is one of representative grass species that is widely used in livestock grazing in wild grasslands at Mongolia region. It has an advantage that it can be used forage for long periods of time and its value as feed is excellent, its importance in maintaining grass resources and expanding the use of grass resources is gradually increasing (Li *et al*., 2006).

The importance of grassland vegetation in the Mongolia region is being emphasized not only as a stable roughage supply source but also for soil preservation. Accordingly, to prevent water quantity reductions caused by the diverse extreme environmental factors in the Mongolia region. The development of new varieties of environmental stress resistant crops by applying molecular breeding technology is an indispensable condition.

Though few reports on plant regeneration system of Siberian wildrye grass through mature embryo, mesocotyl and leaf tip have been published (Li *et al*., 2006). However, since the importance of some of forage has not yet been highlighted compared to other crops (Bai and Qu, 2000; Bettany *et al*., 2003; Lee *et al*., 2007), studies of new variety development using transformation techniques are rare.

Establishment of efficient and highly reproducible regeneration system would greatly influence the efforts of improvement of this grass species through useful genes transfer technology. Therefore, in this study, with a view to the development of new varieties of Siberian wildrye grass applying forage molecular breeding technology, it is intended to establish conditions for callus induction from mature seeds of Siberian wildrye grass.

MATERIALS AND METHODS

Plant material and seed sterilization: Mature seeds of Siberian wildrye grass (*Elymus sibiricus* L.) local *ecotype* were supplied by the Mongolian forage seed producers association. Approximately 2 g of mature seeds were treated for 30 minutes in 50% sulfuric acid (H_2SO_4) and rinsed with distilled water for 1-2 min. After rinsing with distilled water seeds were surface-sterilized by immersion in a solution of 30% (w/v) sodium hypochlorite for 30 min. Two drops of Tween-20 were added. To remove the surfactants, sterilized seeds were rinsed five times with sterile deionized-distilled water and blotted on to a sterile Whatman filter paper.

Callus induction: The callus induction culture medium used to induce calluses from mature seeds was made by adding 0.5 g/L L-proline, 0.5 g/L casamino acid, 30 g/L sucrose and 3 g/L Gelrite to the MS (Murashige and Skoog, 1962) basic culture medium. To investigate the effect of plant growth regulators (PGRs) on callus

induction, the callus induction culture medium added with single or both of 2,4-D (2,4-dichlorophenoxyacetic acid) and BA (6-benzyladenine) was used. Twenty seeds were transferred on each Petri dish (87×15 mm) containing 30 ml of callus induction medium. The cultures were transferred to controlled growth chamber at 24 ± 2ºC under continuous dark. After four weeks callus was removed manually from the germinating shoots and roots, and sub-cultured on the same medium and conditions for two weeks. Callus formation was indicated by the ratios of the numbers of induced calluses to the numbers of seeds sown in percentages and the fresh-weight of the calluses formed from each seed was examined three times and compared.

Plant regeneration: In order to examine appropriate types and concentrations of PGRs for plant regeneration from mature seed derived calluses, six weeks old embryogenic calluses were cut to 3-4 mm diameter sizes and moved to the N6 (Chu *et al*., 1975) medium regeneration culture medium containing 1 mg/L 2,4-D, 3 mg/L BA, 0.5 g/L L-proline, 0.5 g/L casamino acid, 3% sucrose and 3 g/L Gelite, and added with single or both of auxin and cytokinin to be cultured for three weeks in a 24 ± 2^{o}C, 16 h light/8 h dark condition. Then, the calluses were subcultured to a new culture medium in the same composition and cultured for six weeks and the shoots formed in each treated were examined as regenerated individuals. The plant regeneration rates were indicated by the ratios of the numbers of calluses where plants were induced to the numbers of transplanted calluses in percentages. The regenerated shoots were transplanted into a 1/2 MS culture medium to induce root generation to make the shoots differentiate into complete plants. Then, the plants were transplanted to soil and cultivated in greenhouses.

Carbon source: In order to examine the culture effects by type of carbon sources added to the culture medium for inducing calluses from mature seeds and the plant regeneration culture medium as energy sources, sucrose, maltose, glucose or sorbitol at a concentration of 30 g/L were separately added to the callus induction culture medium and the regeneration culture medium and the seeds and calluses were cultured. Then, the callus induction efficiency and plant regeneration rates were examined separately in the same method as mentioned above.

RESULTS AND DISCUSSION

Effect of plant growth regulators on callus induction: Plant tissue culture efficiency differs greatly depending on the types and concentrations of plant growth regulators and substances added to culture media (Somer et al., 2003). The results shown in table 1 depicted that callus induction from the mature seeds was highest at 53.3% when treated with 5 mg/L 2,4-D only and the highest ratio of calluses formed in these had dense tissues. The rate of callus induction from the mature seeds showed a decreasing tendency at lower concentrations. The fresh-weight of calluses formed per seed showed a tendency to increase as the concentration of added 2,4-D increased and was the highest when treated with 5 mg/L 2,4-D at around 42.7 mg.

Besides, the culture effect of 5 mg/L 2,4-D treatment that showed the highest callus induction rate and the culture effect of treatment by 2,4-D mixed with BA were examined. Although callus induction rates decreased a little in general compared to treated with 2,4-D only, the fresh-weight was shown to be the highest at 52.7 mg when 5 mg/L 2,4-D and 0.1 mg/L BA were added in combination. The callus induction rates and fresh-weight decreased at higher BA concentrations.

Table 1: Effect of 2,4-D and BA on callus formation and plant regeneration from mature seed cultures of Siberian wildrye grass

Growth regulator(mg/L)		No. of seeds transferreda	Callus formation (%)	Callus fresh weight per seed (mg)
2,4-D	BA			
0	-	50	0	0
1	-	120	16.6	28±1.0
3	-	120	41.7	35.7±2.1
5	-	120	53.3	42.7±2.1
5	0.1	120	50.8	52.7±2.5
5	0.5	120	40.0	40.3±1.5
5	1	120	31.6	25.7±2.1

[a]Dehusked mature seeds were placed on MS medium containing 3% sucrose, 0.5 g/L L-proline, 0.5 g/L casamino acid and 3 g/L Gelite, and cultured for 6 weeks.

Inclusion of a lower concentration of BA with 2,4-D was found to increase the callus induction frequency, callus quality and its regeneration capability (Dahleen and Bregitzer, 2002; Huang and Wei, 2004). Thus, findings of present investigation were in conformity with above conclusion.

Effect of plant growth regulators on plant regeneration: Plant regeneration efficiency was examined using calluses that were formed in the callus induction culture medium added with 1 mg/L 2,4-D and 3 mg/L BA that showed the best callus induction efficiency and the results are as shown in Table 2.

Among the treated with the combinations of 2,4-D and BA in various concentrations, in the case of 2,4-D, treated with 1 mg/L showed higher regeneration rates than those treated with 2 mg/L in general and in particular, the treated with 1 mg/L 2,4-D and 3 mg/L BA showed the highest plant regeneration rate of 61.1%. However, treated with BA in lower or higher

concentrations decreased regeneration rates. In case of treated with 2 mg/L 2,4-D, no regeneration took place and only active callus proliferation occurred. Therefore, it was considered that for plant regeneration from Siberian wildrye grass seed derived calluses, adding 1 mg/L 2,4-D (low concentration) and 3 mg/L BA would be the most efficient.

Table 2: Effect of 2,4-D and BA on plant regeneration from mature seed culture of Siberian wildrye grass

Growth regulator (mg/L)		Plant regeneration
2,4-D	BA	(%)
1	0.5	18.9
1	1	22.2
1	3	61.1
1	5	42.2
2	0.5	24.4
2	1	21.1
2	3	17.8
2	5	14.4

[a]Calli were transferred to N6 medium containing 3% sucrose, 0.5 g/L L-proline, 0.5 g/L casamino acid and 3 g/L Gelite, and cultured for 6 weeks.

Effects of different carbon sources: In the case of callus induction added with 3% sucrose showed the highest callus induction rate of 56.7%, maltose depicted an induction of 47.5% while added with glucose and sorbitol gave low callus induction rates of 40% and 19.2%, respectively. In case of plant regeneration, added with sucrose showed the highest efficiency of 55%, maltose 51%, glucose 45% and sorbitol showed the lowest efficiency of 21%. In particular, added with sucrose tended to show the highest regeneration rate as well as the largest number of regenerated plants per callus. Therefore, it is considered that, in Siberian wildrye grass seed culture, 3% sucrose would be efficient for callus formation and plant regeneration.

Recently, a result indicating that, in the case of plant regeneration in gramineous plant callus culture, adding BA as cytokinin along with low concentration auxin would improve embryogenic callus induction rates and regeneration rates was reported in the culture of turf-type tall fescue's immatured embryos and mature seeds (Bai and Qu, 2000) and plants such as Kentucky bluegrass (Griffin and Dibble, 1995) and Bermuda grass (Chaudhury & Rongda, 2000).

Table 3: Effect of carbon sources on callus formation and plant regeneration in mature seed cultures of Siberian wildrye grass

Carbon source	No. of seeds transferred[a]	Callus formation (%)	No. of calli transferred	Plant regeneration (%)[b]
Sucrose	120	56.7	100	55.0
Maltose	120	47.5	100	48.0
Glucose	120	40.0	100	41.0
Sorbitol	120	19.2	100	20.0

[a]Dehusked mature seeds were cultured on the MS medium containing 5 mg/L 2,4-D, 0.1 mg/L BA, 0.5 g/L L-proline, 0.5 g/L casamino acid, 3 g/L Gelrite, and cultured for 6 weeks.
[b]Calli were transferred to the N6 medium containing 1 mg/L 2,4-D, 3 mg/L BAP, 0.5 g/L L-proline, 0.5 g/L casamino acid, 4 g/L Gelite, and cultured for 6 weeks.

These results mean that, even if cultured with appropriate growth regulators and culture medium conditions, the effect of carbon sources added into culture media on regeneration rates would be very large (Fieser and Vanzant, 2004).

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 Siberian wildrye grass mature seeds (Figure 1). It is expected that this efficient Siberian wildrye grass regeneration system can be usefully used in developing new varieties of transformed plants using molecular breeding.

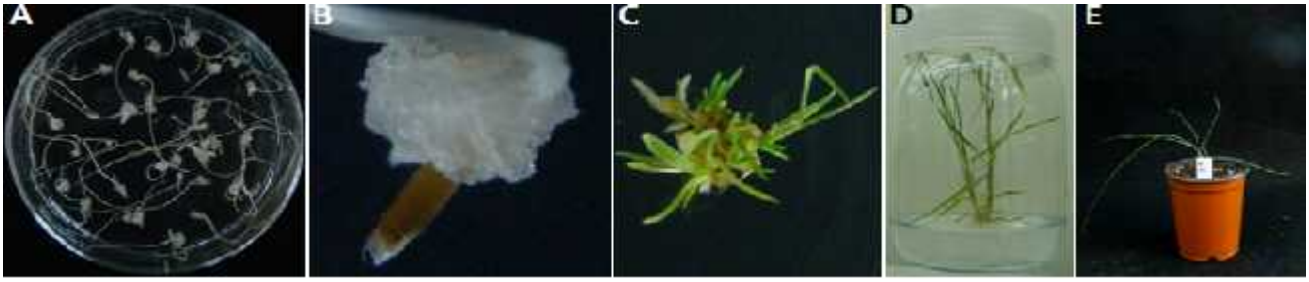

Figure 1: Plant regeneration from mature seed-derived callus of Siberian wildrye grass. A-B, callus induced from mature seeds cultured on the callus induction medium; C-D, Plant regeneration from embryogenic calli in the regeneration medium; E, Whole plant grown in pot

Acknowledgments: This study was supported by 2012 Post Doctoral Course Program of National Institute of

Animal Science, Rural Development Administration, Republic of Korea.

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