

Full Length Research Paper

High frequency plant regeneration from mature seed-derived callus of Italian ryegrass (*Lolium multiflorum*) cultivars

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In the present study, we have developed a high-frequency plant regeneration system for Italian ryegrass via callus culture using mature seeds as explants. Optimal embryogenic callus induction was found to occur in MS medium containing 5 mg l⁻¹ 2,4-D, 0.5 mg l⁻¹ BA, 500 mg l⁻¹ L-proline, 1 g l⁻¹ casein hydrolysate, 30 g l⁻¹ sucrose, 7 mg l⁻¹ AgNO₃, 2 mg l⁻¹ CuSO₄ and solidified with 3 g l⁻¹ Gelrite. The highest regeneration rate was obtained in MS medium containing 1 mg l⁻¹ 2,4-D, 5 mg l⁻¹ BA, 500 mg l⁻¹ L-proline, 1 g l⁻¹ casein hydrolysate, 1 mg l⁻¹ thiamine-HCl, 30 g l⁻¹ sucrose, 7 mg l⁻¹ AgNO₃, 2 mg l⁻¹ CuSO₄ and solidified with 3 g l⁻¹ Gelrite. By using the most effective treatment determined for each parameter, the highest rates of embryogenic callus formation (48.9%) and regeneration (47.6%) were obtained with the Hwasan 101 cultivar. The overall plant regeneration rates of the examined cultivars ranged from 7.5 to 23.2%. Thus, optimization of regeneration frequency using mature seeds as explant material may offer a simple and efficient protocol for Italian ryegrass that may improve molecular breeding of this species.

Key words: Italian ryegrass, *Lolium multiflorum*, embryogenic callus, plant regeneration, growth additives.

INTRODUCTION

Plant tissue culture techniques are an important component of the molecular breeding of plants and can lead to the generation of new plant cultivars in a comparatively short time frame compared to the conventional breeding method. Biotechnological approaches may contribute to the development of improved cultivars for forage crops. Therefore, the use of biotechnological tools, including *in vitro* culture and genetic transformation, may be considered for the improvement of quality and enhancement of resistance to different abiotic or biotic stresses of grass

species (Ye et al., 1997).

Italian ryegrass, also called annual ryegrass, is one of the fastest growing and short-lived perennial grass species widely used for forage. It provides an excellent high quality feed for most classes of livestock and is regarded as a versatile grass species that can be used for pasture, hay, green chop or silage (Hides et al., 1993; Isselstein, 1993). Improvement of cold tolerance of Italian ryegrass is an important breeding objective for enlargement of the cultivation area in Korea (Choi et al., 2008). Establishment of a highly efficient and reproducible regeneration system would thus greatly enhance efforts to improve this grass species through gene transfer technology. Plant regeneration of Italian ryegrass through organogenesis or via somatic embryogenesis has been reported earlier from immature embryos (Dale, 1980), immature inflorescences and nodes (Creemers-Molenaar et al., 1988; Dale et al., 1981), ovules (Kumlehn and Nitzsche, 1995)

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Abbreviations: 2,4-D, 2,4-dichlorophenoxy acetic acid; BA, 6-benzyladenine; MS, Murashige and Skoog (1962) medium.

or by shoot tip culture (Wataru et al., 2004).

A few studies have reported plant regeneration and subsequent regeneration of transgenic Italian ryegrass through the mature seed-derived callus. Mature seeds are widely used as a source of explants in order to initiate *in vitro* cultures in Gramineae family plants for several reasons (Ha et al., 2001; Dong and Qu, 2005; Lee et al., 2006; Lee et al., 2007; Liu et al., 2008). Mature or dry seeds can be used as an important source of explants for *in vitro* culture systems in all seasons and under all conditions. Moreover, dry seeds can easily be handled as explants compared to explants of other tissues. Therefore, recently mature seeds are considered one of the most popular and efficient sources of explants for plant regeneration via callus culture or somatic embryogenesis in Gramineae family plants (Ha et al., 2001; Dong and Qu, 2005; Lee et al., 2006; Lee et al., 2007; Liu et al., 2008).

In this study, we sought to develop an efficient and reproducible regeneration system from mature seed-derived calluses of Italian ryegrass (*Lolium multiflorum*) cultivars for genetic transformation. In the present study, genotypic differences in callus induction, subculture and plant regeneration of Italian ryegrass cultivars were examined.

MATERIALS AND METHODS

Plant materials

Mature seeds of seven Italian ryegrass (*Lolium multiflorum*) cultivars, namely, Kogreen, Kospeed, Kowinearly, Kowinmaster, Hwasan 101, Hwasan 104 and Kowinner, were collected from the Grassland and Forages Research Center, National Institute of Animal Science, Rural Development Administration, Korea and were used as the source of explants.

The clean mature seeds were dehusked by stirring in 50% sulfuric acid for 30 min and rinsed with sterile water. Dehusked seeds were then surface-sterilized with 70% ethanol for 1 min and 30% bleach (5.25% sodium hypochlorite) for 30 min with gentle shaking, followed by three washes with sterile water. Finally, they were blotted with sterile Whatman's filter paper.

Callus induction, subculture and differentiation

The surface-sterilized seeds were placed in MS medium containing MS basal salts and vitamins (Murashige and Skoog, 1962), 500 mg l⁻¹ L-proline, 1 g l⁻¹ casein hydrolysate, 30 g l⁻¹ sucrose, 3 g l⁻¹ Gelite. Different concentrations of plant growth regulators (2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BA)) were applied to each Petri dish (87 × 15 mm). The media pH was adjusted to 5.8 before autoclaving.

The cultures were transferred to controlled growth chambers at 24 ± 2°C for four weeks in the dark. After four weeks, calluses were removed from the germinating shoots and divided into 4 - 6 mm-diameter pieces and sub-cultured every three weeks in the same medium. Callus formation, multiplication and necrosis frequency were observed every three weeks according to callus subculture time.

Cultivar Hwasan 101 was used as the initial model for optimi-

zation of tissue culture conditions. For each of the seven cultivars tested, there were three replicates of 900 seeds. The callus induction frequency was measured as the percentage of seeds that produced a callus.

Plant regeneration

Six-week-old compact embryogenic calli were selected and plated on MS basal medium containing 1 mg l⁻¹ 2,4-D, 5 mg l⁻¹ BA, 1 g l⁻¹ casein hydrolysate, 500 mg l⁻¹ L-proline, 1 mg l⁻¹ thiamine-HCl and 3 g l⁻¹ Gelrite. The cultures were transferred to fresh medium every three weeks and maintained at 24 ± 2°C under a fluorescent light (80 µE m⁻² s⁻¹) at a 16-h photoperiod.

Factors evaluated

Different additives such as AgNO₃ (1 - 10 mg l⁻¹) or CuSO₄ (1 - 5 mg l⁻¹) were added to the medium to determine the best conditions for embryogenic callus formation and high frequency plant regeneration.

Rooting and acclimation of regenerated plants

Regenerated shoots (3-4 cm) were further transferred to rooting medium consisting of half-strength MS with 30 g l⁻¹ sucrose. Cultures for rooting were kept under fluorescent light (80 µE m⁻² s⁻¹) at 24 ± 2°C with a 16 h photoperiod for a couple of weeks. Plantlets with well developed roots were removed carefully from the culture medium and placed into pots for 3 - 4 weeks in a growth chamber at 22°C day/20°C night cycle under a 16 h photoperiod. Rooted plantlets were transferred to greenhouse conditions.

RESULTS AND DISCUSSION

Effect of growth regulators on callus induction

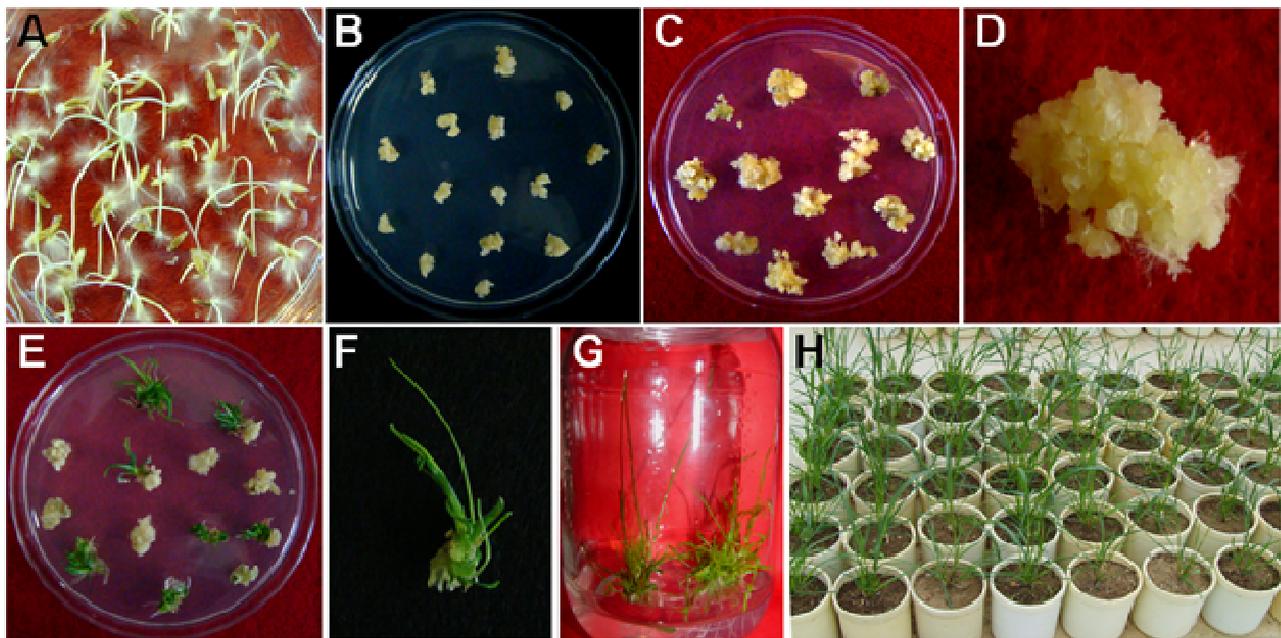
To examine the effect of auxin on callus induction, 2, 4-D was used at different concentrations (1 to 5 mg l⁻¹) (Table 1). Calluses appeared from mature seeds within 5-10 days on the callus induction medium (Figure 1A). The primary callus induction frequency varied from 63.8 to 83.2% after a culture period of four weeks. The primary calluses were then separated and transferred to the subculture medium (Figure 1B). The embryogenic calluses were obtained after two passages subculture, (Figures 1C and D) and subsequently transferred to the regeneration medium.

Callus induction frequency was increased along with the increase of the concentration of 2,4-D up to 5 mg l⁻¹ (Table 1), however a concentration of 2,4-D more than 5 mg/l in the basal medium showed a tendency to decrease of callus proliferation. Among the concentrations tested, 5 mg l⁻¹ 2, 4-D showed the highest induction of embryogenic calluses (33.7%) in the presence of callus induction medium.

Our results are consistent with studies that have demonstrated that compared to other auxins, 2, 4-D is

Table 1. Effect of 2,4-D on callus formation and plant regeneration from mature seed cultures of Italian ryegrass (cv. Hwasan 101).

2,4-D Concentration	No. of seeds transferred	Primary callus induction (%)	Embryogenic callus induction (%)
0	100	0	0
1	900	63.8 ± 6.9	15.4 ± 3.8
3	900	70.1 ± 3.0	24.2 ± 4.7
5	900	83.2 ± 1.3	33.7 ± 4.3
7	900	78.3 ± 2.6	30.9 ± 2.8
9	900	74.4 ± 6.4	26.6 ± 1.7

**Figure 1.** Primary callus induction, secondary growth of the callus and plant regeneration from dehusked mature seeds of Italian ryegrass (cv. Hwasan 101). (A) Calluses appeared from the mature seeds; (B) Germinating shoots and roots were removed from the calluses; (C, D) Compact nodular embryogenic calluses; (E, F) Regeneration of shoots four weeks after plating; (G) Regenerated plants in the rooting medium; (H) Plantlets growing in the greenhouse.

more effective for callus induction in grass species including Italian rye grass (Wataru et al., 2004), orchard-grass (Lee et al., 2006), tall fescue (Lee et al., 2007), centipedegrass (Liu et al., 2008).

Moreover, our results also showed that cytokinin (BA) enhanced callus induction frequency up to 39.1% (Table 2). Among the concentrations and combinations of 2,4-D and BA examined, MS medium fortified with 5 mg l⁻¹ of 2,4-D and 0.5 mg l⁻¹ of BA was found to be superior for embryogenic callus induction (Table 2). It has also been suggested in several studies that a higher concentration of auxin in combination with a low level of cytokinin is superior for plant regeneration via callus formation (Cho et al., 1998; Chaudhury and Qu, 2000; Zhong et al.,

1991; Liu et al., 2008).

Thus, combination of the optimum levels of auxin and cytokinin for callus induction and regeneration showed far better results, which were statistically significant compared to the effects of single hormones.

Effects of additives on callus induction and plant regeneration

Additional uses of several additives such as AgNO₃ and CuSO₄ combined with the hormones in the tissue culture medium have been shown to promote callus induction and regeneration in several plants (Adkins et al., 1993;

Table 2. Effect of BA on plant regeneration from mature seed-derived calluses of Italian ryegrass (cv. Hwasan 101).

2,4-D	BA	No. of seeds transferred	Embryogenic callus induction (%)	No. of calli transferred	Plant regeneration (%)
5	0	900	32.7 ± 1.2	225	30.2 ± 3.4
5	0.1	900	34.4 ± 3.6	225	31.1 ± 2.0
5	0.5	900	39.1 ± 3.1	225	40.4 ± 3.4
5	1.0	900	38.2 ± 3.5	225	37.3 ± 6.9
5	1.5	900	22.9 ± 3.0	225	24.4 ± 2.8

Table 3. Effect of AgNO₃ and CuSO₄ concentrations on callus formation and plant regeneration from mature seed cultures of Italian ryegrass (cv. Hwasan 101).

Index	Concentration (mg l ⁻¹)	Embryogenic callus induction (%)	Plant regeneration (%)
MS	0	37.7 ± 3.5	41.3 ± 4.0
AgNO ₃	1	38.2 ± 3.4	40.9 ± 2.0
	3	39.3 ± 1.9	41.8 ± 7.8
	5	42.4 ± 3.7	43.6 ± 3.8
	7	45.4 ± 3.0	45.8 ± 5.0
	10	43.6 ± 3.4	44.9 ± 4.1
CuSO ₄	1	39.6 ± 2.2	44.0 ± 4.8
	2	44.8 ± 1.0	44.9 ± 4.1
	3	42.1 ± 0.8	42.2 ± 5.4
	5	36.6 ± 1.9	41.8 ± 5.4
AgNO ₃ + CuSO ₄	7+2	48.4 ± 3.8	46.2 ± 3.4

Fei et al., 2000; Dahleen, 1995; Tahiliani and Kothari, 2004; Sahrawat and Chand, 1999). We therefore further tested the effects of various kinds of plant growth additives, such as AgNO₃ and CuSO₄. These were added individually or in combination to the medium in order to demonstrate their effects on callus induction and plant regeneration of Italian ryegrass (Table 3).

The results showed that, together with the optimum growth regulators, additional of AgNO₃ and CuSO₄ at different concentrations significantly improved callus proliferation and plant regeneration rates (Table 3).

Among the additives used, 7 mg l⁻¹ of AgNO₃ significantly improved the callus proliferation rate from 37.7 to 45.4% and regeneration ability from 41.3 to 45.8%. However, higher concentrations of AgNO₃ showed callus browning and a decrease of embryogenic callus proliferation and regeneration rates. Callus browning is one of the major problems in *in vitro* culture. The effects of AgNO₃ on callus induction and regeneration have been previously studied (Adkins et al., 1993; Fei et al., 2000).

Several studies have shown that the use of CuSO₄ in the basal medium has a stimulatory effect on callus induction, proliferation, somatic embryogenesis and rege-

neration of monocotyledonous plants (Dahleen, 1995; Tahiliani and Kothari, 2004; Sahrawat and Chand, 1999). In the present study we found that the effect of CuSO₄ on callus induction and regeneration was much lower than that of AgNO₃. Interestingly, the combination of 7 mg l⁻¹ AgNO₃ and 2 mg l⁻¹ CuSO₄ resulted in maximum induction of embryogenic callus proliferation (48.4%) and shoot regeneration (46.2%).

Regenerated plantlets (Figure 1E and F) were transferred to the rooting medium. Rooted plantlets (Figure 1G) were transferred to a controlled growth chamber and subsequently to greenhouse (Figure 1H).

Effects of genotypic variation on callus induction and plant regeneration

Similar to earlier reports (Chaudhury and Qu, 2000; Bai and Qu, 2000; Zale et al., 2004), our results also revealed that callus induction and plantlet regeneration frequency are dependent on genotypic variation among the seven cultivars. When the optimum conditions for callus induction and regeneration were applied to the

Table 4. Effect of cultivars on callus formation and plant regeneration from mature seed culture of Italian ryegrass.

Cultivar	Embryogenic callus induction (%) (A)	Plant Regeneration (%) (B)	Overall Plant Regeneration (%) (AxB)
Kogreen	37.8 ± 2.2	32.0 ± 6.1	12.1
Kospeed	44.6 ± 2.4	41.8 ± 4.3	18.6
Kowinearly	45.8 ± 3.6	35.1 ± 7.6	16.1
Kowinmaster	34.3 ± 3.1	36.9 ± 6.8	12.7
Hwasan 101	48.9 ± 1.2	47.6 ± 5.4	23.2
Hwasan 104	24.0 ± 5.1	35.6 ± 7.8	8.5
Kowinner	30.8 ± 2.8	24.4 ± 4.7	7.5

other cultivars, considerable genotypic variation was observed in both callus induction and plant regeneration (Table 4). Callus induction and shoot regeneration frequency ranged from 24 to 48.9% and from 24.4 to 47.6%, respectively. Cultivar Hwasan 101 showed relatively higher callus induction (48.4%) and regeneration (46.2%) frequencies than the other six cultivars. In contrast, cultivar Kowinner showed the lowest shoot regeneration frequency 24.4% compared to the other cultivars.

Variations in callus induction and regeneration ability among the tested cultivars indicated that differences in the responses were due to differences among the genotypes. Genotypic variations in *in vitro* culture system are frequently observed in grass species (Chaudhury and Qu, 2000; Bai and Qu, 2000; Zale et al., 2004). In summary, we have optimized a high frequency callus induction and regeneration protocol for Italian ryegrass, which was shown to be a simple and highly reproducible approach with potential applicability for molecular breeding of Italian ryegrass by the production of transgenic plants.

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