High Efficiency of Plant Regeneration from Seed-Derived Callus of Zoysiagrass cv. Zenith

Ming Liang Chai, Jong Min Lee, Doo Hwan Kim*

Dept. of Horticultural Science, Konkuk University, Seoul 143-701, Korea

Zoysiagrass japonica의 효율적인 재분화체계에 관한 연구

Ming Liang Chai · 이종민 · 김두환*

건국대학교 농업생명과학대학 원예과학과

ABSTRACT

The development of a protocol for high efficiency of embryogenic callus separation, maintenance and plant regeneration from the seeds of zoysiagrass cv. Zenith was studied. Embryogenic callus ratio is absolutely determined by genotype, but by adding high concentration of copper into medium, changing light condition and maintaining callus on initial induction medium for 8~10 weeks, embryogenic callus can be easily distinguished and its growth can be promoted. There were significant differences among selected callus lines (each from one seed) according to their growth rates and regeneration percentages. Callus pre-treatment with activated charcoal inhibited callus growth, increased the level of precocious germination during culture and promoted shoot cluster formation after transfer to regeneration medium. For long-term callus maintenance, N6AA medium was better than MS medium, because the former inhibited non-embryogenic callus formation and kept vigor of embryogenic callus. The best callus lines Z-(5) has been successfully used for transformation and somaclonal variation selection in our laboratory.

Key words: zoysiagrass, Zenith, embryogenic callus, regeneration

INTRODUCTION

Zoysiagrass is one of the most commonly used warm-season turfgrasses throughout the warm season and transition zones. It has several attractive characteristics for a turf, such as traffic, drought and shade tolerance. Though it is generally tolerant to diseases and insects, it is highly susceptible to some diseases and insects such as large patch and hunting billbug. With its increase in the commercial utilization, the demand for genetically modified cultivars with the improved tolerance increases.

*corresponding author

Modern biotechnological techniques such as somaclonal variation and transformation have been recognized as useful tools for the development of improved plants. In zoysiagrass, callus induction (Al-Khayri et al., 1989; Inukuma et al., 1996; Kim et al., 1997) and transformation by particle bombardment (Ahn et al., 1998) and PEG-mediated direct gene transfer (Inukuma et al, 1998) have been reported. However, the lack of regeneration potential from callus in zoysiagrass limits the application of biotechnological techniques for the genetical improvement. The application of tissue culture to zoysiagrass improvement requires identifying the genotypes capable of regenerating entire plants (Varge and Badea, 1992).

The objectives of this study were 1) to evaluate the effect of medium on callus induction and maintenance; 2) to determine the period for separating embryogenic part from its initial callus and to ascertain the characteristics of embryogenic callus; 3) to establish suspension culture; 4) to evaluate pre-treatment of activated charcoal on callus growth and subsequent regeneration in zoysiagrass cv. Zenith.

MATERIALS AND METHODS

The seeds of zoysiagrass cv. Zenith were used in this experiment. The hulled seeds were surface sterilized with 70% ethanol and 0.1% $HgCl_2$ for 1 and 20 minutes, respectively. After rinsing repeated with sterile water, the seeds were placed on disposable plastic petri-dishes (90 mm in diameter) containing 25 ml of medium solidified with 0.3 % phytagel (except for phytagel concentration test). For the initiation of callus, MS (Murashige and Skoog, 1962), N6AA (Asano and Sugiura, 1990) and NP (Islam et al., 1998) media were used.

According to experimental design, the media were supplemented with 2,4-D (2,4-dichrolophenoxyacetic acid), BA (6-Benzylaminopurine), copper and activated charcoal and adjusted to pH 5.8 (except for medium pH test) prior to autoclave at 121 ℃ for 15 minutes. Seeds were incubated in culture room in the dark or light (16h, 1000 lux) for callus induction. Embryogenic calli were maintained by subculturing at monthly intervals. Plant regeneration was estimated under light condition about 2000 lux. Suspension cultures were maintained in liquid medium of N6AA+2 mg/L or MS+2 mg/L 2,4-D in the shaker incubator 120 rpm at 28 ℃, subculturing at 5 to 7 days intervals. After 8~10 weeks, the calli on callus induction media were weighed and then transferred to basal MS medium to regenerate. Three weeks later, embryogenic callus was determined according to regenerating style (i.e. embryogenesis). Callus of Z-(1) and Z-(5) was first pre-treated on MS+2 mg/L 2,4-D containing 0, 0.1, 0.5, 1.0, 2.0 % activated charcoal for 1 month, and then checked for regeneration on MS medium after 1 and 2 weeks.

RESULTS AND DISCUSSION

Callus induction and embryogenic callus separation

Callus was induced from any kind of MS, N6AA and NP basal medium supplemented with 2 or 4 mg/L 2,4-D. Usually, almost all the seeds that geminated formed calli. Different basal medium influenced the ratio of callus induction and callus growth (size), but had no significant influence on embryogenic callus formation. About 1 % of the seeds formed embryogenic callus in any kind of media (data not shown).

Our previous experiments showed that supplement of thiamine HCl (5, 10, 20, 30 mg/L), L-proline (10, 20, 40, 70, 100 mM) to MS basal medium had no significant promotion to callus induction and embryogenic callus formation (data not shown). Those results did not agree to some reports (Asano, 1989; Kim et al., 1997). And also, the same situation existed in callus induction at different temperatures (28, 31, 34 ℃) either in dark or in light condition.

The pH of the medium and the concentration of solidifying agent phytagel also had no significant function to embryogenic callus induction (data not shown), although the callus induction ratio varied (Table 1, and 2). But in light condition, callus ratio increased and embryogenic section could be easily distinguished and separated (Table 1, 2 and 3).

It was noticed that medium supplemented with BA and high concentration of copper (50-1000 times as the basal MS medium) increased production of regenerable green tissue for transformation in barley (Cho, et al., 1998). In our experiment (Table 3), 0.1 mg/L BA combined with 100X copper promoted distinguishable embryogenic part, although no increase in embryogenic callus induction was observed.

From the above experiments, and our previous observations (data not shown) on callus induction from zoysiagrass cv. Sunrise and Common, we come to a conclusion that

Table 1. Effect of medium pH on callus induction

Medium pH	No. of inoculated seeds	light condition	No. of callus (%)	No. of organ-ogenesis (%)	E.C. distinguishing
5.0	400	dark	172 (43.00)	74 (18.50)	+
	160	light	72 (45.00)	41 (25.60)	++
5.4	320	dark	132 (41.25)	63 (19.69)	+
	320	light	137 (42.80)	68 (21.25)	++
5.8	320	dark	131 (40.94)	49 (15.31)	+
	320	light	159 (49.70)	93 (29.06)	++
6.2	400	dark	159 (39.75)	78 (19.50)	+
	240	light	112 (46.70)	55 (22.92)	++
6.6	320	dark	128 (40.00)	53 (16.56)	+
	320	light	153 (47.81)	81 (25.31)	++

Basal medium is MS+2 mg/L 2,4-D+0.1 mg/L BA+100X Cu.
E.C : embryogenic callus

Table 2. Effect of phytagel concentration on callus induction

Phytagel concentration (%)	No. of inoculated seeds	Light condition	No. of callus (%)	No. of organogenesis	E.C. distinguishing
0.2	320	dark	146 (45.63)	55 (17.19)	+
	320	light	168 (52.50)	71 (22.19)	++
0.25	320	dark	126 (39.38)	55 (17.19)	+
	320	light	141 (44.06)	54 (16.88)	++
0.30	320	dark	132 (41.25)	60 (18.75)	+
	320	light	164 (51.25)	68 (21.25)	++
0.35	320	dark	113 (35.31)	60 (18.75)	+
	320	light	133 (41.56)	65 (20.31)	++
0.40	320	dark	112 (35.00)	47 (14.69)	+
	320	light	142 (44.38)	68 (21.25)	++

Basal medium is MS+2 mg/L 2,4-D+0.1 mg/L BA+100X Cu

Table 3. Effect of copper concentration on callus induction

Basal medium supplement	No. of inoculated seeds	Light condition	No. of callus (%)	No. of organ-ogenesis (%)	E.C. distinguishing
0	320	dark	135 (42.19)	71 (22.19)	+
	320	light	154 (48.13)	82 (25.63)	++
0.1 mg/L BA	320	dark	126 (39.38)	64 (20.00)	+
	320	light	136 (42.50)	96 (30.00)	++
0.1 mg/L BA +10X Cu	320	dark	128 (40.00)	66 (20.63)	+
	320	light	146 (45.63)	88 (27.50)	++
0.1 mg/L BA +100X Cu	320	dark	126 (39.38)	62 (19.38)	+
	320	light	146 (45.63)	91 (28.44)	+++
0.1 mg/L BA +500X Cu	240	dark	99 (41.24)	45 (18.75)	+
	320	light	153 (47.81)	89 (27.81)	++

Basal medium is MS+2 mg/L 2,4-D

genotype greatly influences the ratio and quality of embryogenic callus. But by modifying medium and changing culture environment, embryogenic calli can be easily distinguishable and growth of embryogenic callus can be improved. In our condition, 'Zenith' was much superior to 'Sunrise' or 'Common'. Fifteen embryogenic callus lines (each from one seed) were selected from about 30000 hulled seeds of 'Zenith' with the characters of high regeneration to normal plants (20 to 70 %), and fast growing during subculture.

The appropriate time for separating embryogenic part from its initial callus was around 8-10 weeks after initiation of callus induction when the medium was nearly exhausted. Under such water and nutrition stress, big size of embryogenic callus section connecting with its original callus can be distinguished and separated easily. And also during this stage embryogenesis or organogenesis can be detected. The ratio of organogenesis was much higher in our experiments, from about 15 % in dark to more than 20 % in light (Table 1, 2, and 3). This situation is the same as that in some other plants. Cure et al.

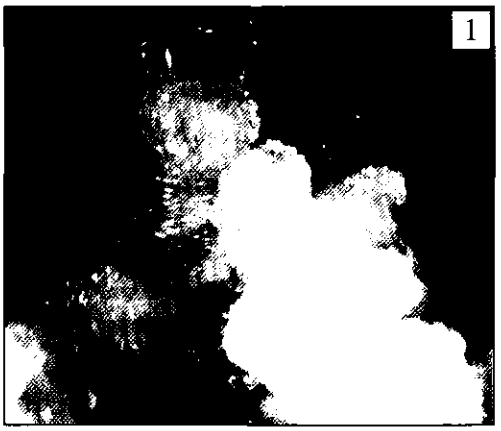

Fig 1. Embryogenic callus of Z-(5)

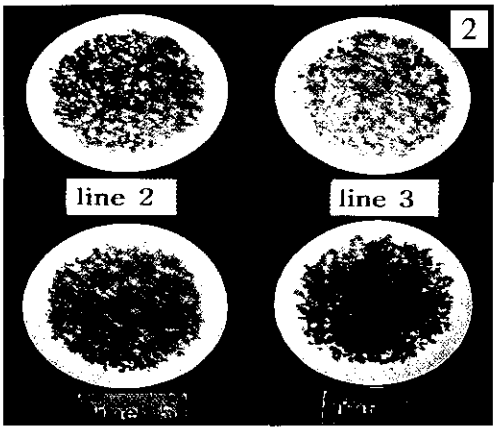

Fig.2. Callus suspension cultures of 'Zenith' callus lines

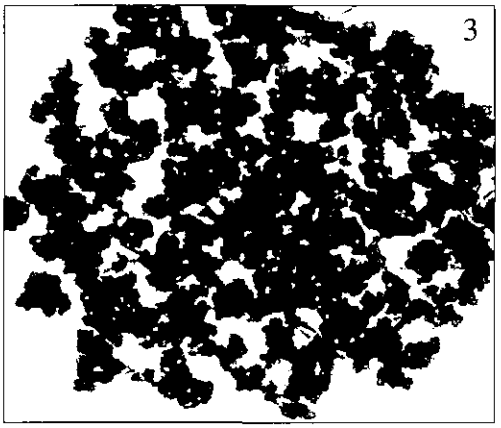

Fig. 3. shoot clusters regenerated from embryogenic callus of Z-(5)

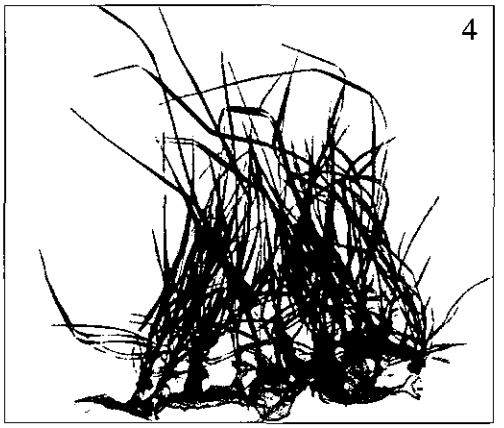

Fig. 4. Plants from shoot clusters of Z-(5)

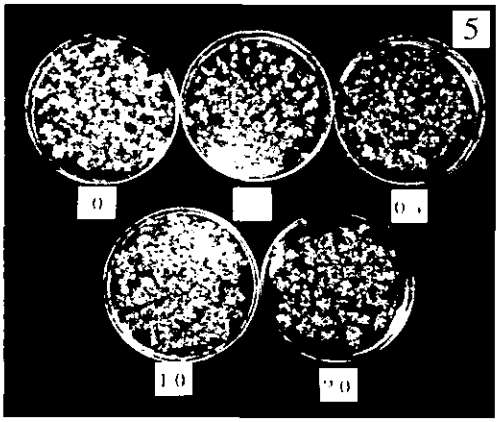

Fig. 5. Effect of activated charcoal pre-treatment on the regeneration of embryogenic callus from Z-(5).

(1978) found that in most cases root and shoot formation is induced from the existing organ-like structure (organogenesis) and not from undifferentiated tissue (embryogenesis) on callus induction in rice, wheat and oat. In zoysiagrass, embryogenic callus is yellow or yellowish, granulated (nodular), compact (hard) or friable (Fig. 1). They usually had almost the same small size, especially after suspension culture (Fig. 2). Non-embryogenic callus is usually white, sometimes yellow to yellowish, tran-

slucent, large and sticky accompanied with high water content. On regeneration medium, relatively high ratio of normal green shoot clusters were formed from our selected embryogenenic callus lines (Fig. 3). Shoot clusters grew into plants in MS medium (Fig. 4).

Callus maintenance

The capacity of embryogenic calli for plant regeneration usually decreases after long-term subcultures. Embryogenic callus easily changes into non-embrogenic callus, so it is important to separate embryogenic part of callus and moving it to new medium during subculture. MS and N6AA media are commonly used in our experiments, and callus growth among lines was different between the two media, but N6AA has shown to inhibit non-embryogenic callus growth and resistant to hairy roots formation. Such function was even more clear in Z-(1) (Table 4).

For long-term embryogenic callus cultures of red fescue (*Festuca rubra* L.), pre-treatment of maintenance media with activated charcoal before regeneration increased the level of precocious germination during culture and significantly increased shoot and root formation after transfer to regeneration media (Ousama et al., 1988). Similar results were found in our experiment. Charcoal inhibited callus growth, promoted precocious germination and also increased regeneration (Fig. 5, 6, and 7). Precocious germination was very typical at the concentrations of 1.0 % and 2.0 % of activated charcoal. As the result, some calli formed hairy roots, so the weights of calli in these two treatments were heavier as compared with 0.5% treatment.

The increased regeneration capacity induced by activated charcoal pre-treatment was most likely due to the absorption of inhibiting substances characteristic of older cultures as well as the absorption and/or reduction of 2,4-D activity (Ousama et al., 1988).

Table 4. Effect of medium on callus growth and embryogenic callus formation of 4 'Zenith' callus lines during subculture period [a]

Line	Medium	Growth rate[b]	Embryogenic callus(%)[c]
Z-(1)	MS+2,4-D 2mg/L	7.7	40.6
	N6AA+2,4-D 2mg/L	3.0	74.4
Z-(2)	MS+2,4-D 2mg/L	3.5	78.2
	N6AA+2,4-D 2mg/L	4.3	77.4
Z-(3)	MS+2,4-D 2mg/L	2.3	57.0
	N6AA+2,4-D 2mg/L	2.9	57.8
Z-(5)	MS+2,4-D 2mg/L	8.0	57.6
	N6AA+2,4-D 2mg/L	4.9	64.5

[a]Each treatment has 4 replications, each replication(one petri-dish, 9 cm in diameter) contains 0.33±0.06g of callus.
[b]Weight of final callus/weight of initial callus.
[c](Weight of embryogenic part of final callus / weight of final callus)×100

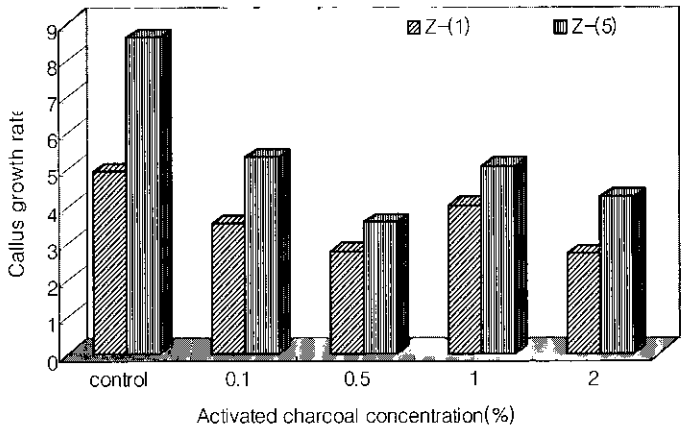

Fig. 6. Effect of activated charcoal pre-treatment on the regeneration of embryogenic callus from Z-(5).
*Each treatment contains 4 petri-dishes, each dishes contains 0.35±0.07g of callus
**Callus growth rate= final weight of callus/initial weight of callus

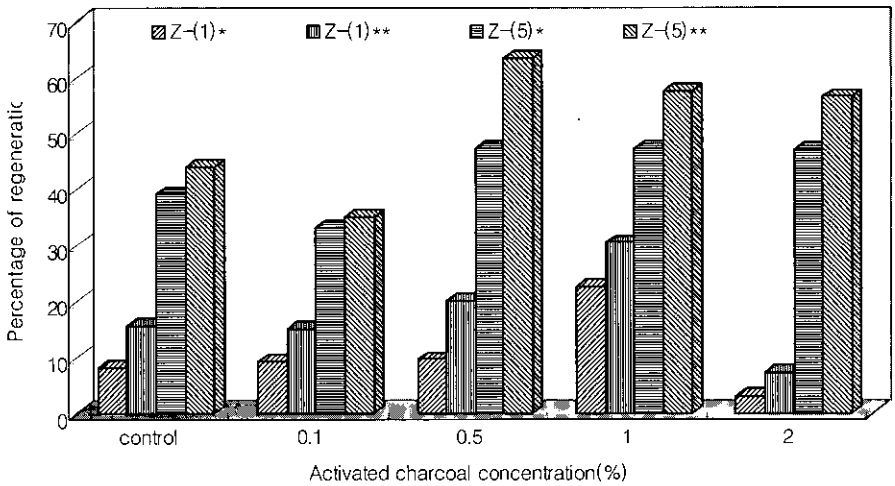

Fig. 7. Effect of activated charcoal on the callus growth of Z-(1) and Z-(5).
Each treatment contains 384±75 pieces of calli.
*: 1 week in MS medium; **: 2 weeks in MS medium.

Literature Cited

Ahn, B. J., and E. A. Lee. 1998. Particle bombardment-mediated transformation of Korean lawngrass (*Zoysia japonica*). Annual Meeting of Korean Society of Plant Tissue Culture and Joint Workshop for Plant Biotechnology Between Korea and Iseral. p:35.

Al-Khayri, J., F. H. Huang, L. F. Thompson, and J. W. King. 1989. Plant regeneration

of Zoysiagrass from embryo-derived callus. Crop Sci. 29:1324-1325.

Asano, Y. 1989. Somatic embryogenesis and protoplast culture in Japanese lawngrass (*Zoysia japonica*). Plant Cell Rep. 8:141-143.

Cho, M. J., Jiang W. and Lemaux P. G. 1998. Transformation of recalcitrant genotypes: improvement in regenerability and decreased albinism. Plant Sci. 138:229-244.

Cure, W. W., and R. L. Mott. 1978. A comparative anatomical study of organogenesis in cultured tissues of maize, wheat, and oats, Physiol. Plant. 42:91-96.

Inukuma, C. Sugiua K., Cho C., Okawara R., and Kaneko S. 1996. Plant regeneration from protoplasts of Japanese lawngrass. Plant Cell Reports. 15:737-741.

Inokuma, C. Sugiura K., Imaizumi N., and Cho C. 1998. Transgenic Japanese lawngrass (*Zoysia japonica* Steud.) plants regenerated from protoplasts. Plant Cell Reports. 17:334-338.

Islam M. O., S. Ichihashi, and S. Matsui. 1998. Control of growth and development of protocorm like body derived from callus by carbon sources in *Phalaenopsis*. Plant Biotechnology. 15:183-187.

Kim, J. B., S. J. Park and D. H. Kim. 1997. Callus induction and plant regeneration from stolon in Zoysiagrass. Korean Journal of Turfgrsass Socciety. 11:311-320.

Murashige T., and Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Ousama, M. F. Zagbmout and William A. Torello. 1998. Enhanced regeneration in long-term callus cultures of red fescue by pre-treatment with activated charcoal. HortScience. 23:615-616.

요 약

한국잔디류인 'Zenith' 종자로부터 캘러스를 유도하였다. 캘러스 발생 8~10주 후 생장률과 재분화율을 고려하여 배발생 캘러스 5개의 영양계(각각의 영양계는 하나의 종자에서 유래)를 선발하였다. 이전에 수행된 Zoysia japonica 'Sunrise'나 'Common' 캘러스 유도 실험과 비교한 결과 유전자형이 질좋은 배발생 캘러스를 유도하는 데 결정적인 요인임을 알 수 있었다. 활성탄이 첨가된 배지에서 캘러스를 유지한 결과 활성탄의 첨가가 캘러스 생장을 억제시키고 조기 재분화를 촉진시켰다. 또한 재분화 배지로 옮긴 후에 shoot cluster 형성을 촉진시키는 결과를 보였다. N6AA 배지가 MS 배지보다 비배발생 캘러스 형성을 억제시키고 배발생 캘러스 활성을 유지시킴을 알 수 있었다.