Embryogenic cell suspension culture and plant regeneration in zoysiagrass (*Zoysia japonica* Steud)

Wenjuan Fang[1], Liebao Han[1]*, Chunhui Qi[1] and Deying Li[2]*

[1]Key Laboratory for Silviculture and Conservation of Ministry of Education; Institute of Turfgrass Science, Beijing Forestry University, Beijing 100083, China

[2]Department of Plant Sciences, North Dakota State University, Fargo, ND 58108, USA

한국들잔디 배아세포의 부유배양과 식물체 재생

팽왼주안[1] · 한리바우[1] · 퀴충휘[1] · 리다이엥[2]* · 박태윤[3]

[1]베이징임업대학교 잔디연구소, [2]노스다코다대학교 식물학과, [3]연세대학교 생명과학기술학부

ABSTRACT

Zoysiagrass (*Zoysia japonica* Steud) is a warm season turfgrass species widely used for sports field and golf courses. Many cultivars are propagated through vegetative methods. This study was conducted to develop an optimum culture medium and culture conditions for embryogenic callus induction and plant regeneration, and to establish a cell suspension culture system for use in zoysiagrass breeding and propagation. The results indicated that adding Cu^{++} at 2.5 mg L^{-1} to the induction medium was optimum for callus induction. Increasing the numbers of sub-culture cycles improved the quality of calli. The optimum dosage for cell suspension culture ranged from 2.5 to 10 mL. The embryogenic callus suspension used in this study had a plant regeneration rate of 58%.

Key words: turfgrass, breeding, embryo, germplasm

Abbreviations: BA: 6-benzyladenine, PCV: packed cell volume.

*Corresponding author. Tel : +1-701-793-4952
E-mail : deying.li@ndsu.edu
Received : Aug., 15, 2009, Revised : Oct., 12, 2009, Accepted : Nov., 30, 2009

INTRODUCTION

Zoysiagrass (*Zoysia japonica* Steud) is a warm season turfgrass species widely used in sports fields, golf course, and parks. In recent years, there has been an increasing interest in zoysiagrass because of its tolerance to biotic and abiotic stresses, and excellent adaptation to low soil fertility (Inokuma et al., 1997, 1998). Zoysiagrass has been considered as a low or minimum maintenance turfgrass species (Brede, 2000). Characteristics needing to be improvement include cold tolerance, color, and texture (Engelke and Anderson, 2003). Traditional breeding methods have limited success due to narrow genetic variations in the germplasm and inter-specific barriers. Transgenic technology has been identified as an efficient way to improve zoysiagrass quality and stress tolerance (Asano, 1989; Ge et al., 2006; Toyama et al., 2003; Wang et al., 2001). Ge et al. (2006) achieved transgenic zoysiagrass plants using *Agrobacterium*-mediated transformation. Tissue and cell culture is an important step duringgenetic transformation. Establishment of an efficient sporophytic plant regeneration system is critical to a molecular breeding program. Al-kharyi et al. (1989) reported a method of regenerating zoysiagrass plant from embryo-derived callus. Inokuma et al. (1997) regenerated a zoysiagrass plant from cell suspensions. It was the first successful case of plant regeneration from protoplasts isolated from the embryogenic suspension cell line in Japanese lawngrass (zoysiagrass). However, the rate of colony formation from protoplasts was very low (0.15%).

Zoysiagrass is one of the grass species which develops different forms of calli from mature embryo by tissue culture. One of the forms of callusis soft and looks water-soaked, and does not have regeneration ability (Koich, et al., 2002). Yu et al (2001) reported that high concentration of C^{++} could selectively eliminate water-soaked calli and keep the soft and friable calli, which aremore suitable for cell suspension. Hu et al. (2003) reported $CuSO_4 \cdot 5H_2O$ at 2.5 mg $L^{-1}$was selectively killing calli after 10 d of culture and those survived turned brown, and after two generations of culture, the calli became loose and bright yellow in color.

The objective of this study was to evaluate medium composition, especially Cu^{++} concentration, and the cultural conditions for callus induction and plant regeneration.

Materials and Methods

Two grams of seeds of zoysiagrass 'Zenith' were added to a 50-mL centrifuge tube containing 37 mL concentrated sodium hypochlorite (10% chloride) and two drops of

Tween-20, stirred continuously for 60 min, and then rinsed five times with sterile distilled water. After decaping the hulls in the suspension, the caryopsis were soaked in sterile distilled water for three days at 4°C with daily water replacement. The imbibed seeds were surface sterilized again in sodium hypochlorite solution for 20 min, rinsed with sterile distilled water three times, and then cultured on MS_2 medium for callus induction in the dark at 28°C. The medium was prepared by supplementing 2 mg L^{-1} of 2, 4-D, 0.3 % agar and 3% sucrose to basic Murashige and Skoog medium (Murashige and Skoog, 1962), adjusting to pH 5.8 with 0.1 M NaOH, and autoclaving at 121ºC for 20 min. After four weeks of culture, the induced calli were sub-cultured monthly. Two months later the calli were classified into three types: Type I was embryogenic with bright yellow color and firm texture; type II callus was friable without secretion; type III callus was white, highly friable, and non-embryogenic. Type III callus was discarded and types I and II were further cultured.

Type II calli were cultured in MS_2 liquid medium at a concentration of 3 to 5 g fresh tissue per 100 mL culture medium in 250-mL conical flasks keptin the dark at 27°C and rotating at 100 rpm. During the first month of culture, half the volume of medium was replaced with new medium every three to fourdays. One month later, three fourths of volume of medium was replaced by new medium every six days. Cell concentrations were monitored by measuring the optical density (O.D.) at 600 nm using a spectrophotometer. Five cultural dosages of cell suspension measured as packed cell volume (PCV), that is, 2.5, 5, 7, 10, and 13 mL per 100 mL of medium, and four concentrations of Cu^{++} at 0, 1.25, 2.5, and 5.0 mg L^{-1} in the MS_2 medium were tested in the study. PCV and pH in the medium were measured once every two days. After 10 days of culture, the suspension aggregates were filtered with 120-㎛ sieve and the through falls were cultured for 3 additional days (volume of cell aggregates to volume of medium was 10:100). The experimental design was completely randomized with 5 replications.

Light yellow and relatively clear callus suspensions of similar sizes (about 2 mm diameter) were dried by blotting with sterile filter paper. The calli were then cultured in alternative cultural cycles with regular MS_2 and on modified MS_2 medium with 6-BA at 0.1 mg L^{-1} to promote cell division and maintain embryogenic potential. Regeneration rate is expressedas number of regenerated calli divided by the total number of all calli.

Results and Discussion

Germination and calli generation from the caryopsis started 10 days after the initial induction culture. Type II embryogenic calli increased as the number of sub culture cycle increased (Table 1). Overall, the rate of type II calli was relatively low (5% with 4 sub-culture cycle (Table 2).

Table 1. Influence of numbers of sub-culture cycles on callus quality of 'Zenith' zoysiagrass

Number of cycles	Type I callus†	Type II callus	Type III callus
		%	
1	1.2c‡	0c	98.8a
2	3.1b	1.0b	95.9ab
3	7.6a	4.8a	87.6b
4	7.8a	5.0a	87.2b

† Type I calli were bright yellow with firm texture and were embryogenic; type II calli were friable and did not secrete liquid; type III calli were white and highly friable and were non-embryogenic.

‡ Values followed by same letters within each column are not significantly at 0.05 levels.

Table 2. Influence of Cu^{++} concentrations on the embryonic callus induction of 'Zenith' zoysiagrass

Cu^{++} in MS2	Embryonic callus	Browning callus	Regeneration†
mg L^{-1}		%	
0	9.33a‡	2.67c	0c
1.25	4.00b	37.00b	5.00b
2.50	10.99a	32.97b	15.88a
5.00	2.13c	63.04a	19.56a

† Number of differentiated calli /number of total starting calli. The means are from 4 plates.

† Values followed by same letters within each column are not significantly at 0.05 levels.

Type I gradually converted to type II in subsequent 20-d sub-culture cycles after discarding the Type III callus. Cell suspension form Type II calli grew exponentially over time (Fig. 1). The calli were at lag stage at the first two days of culture, and then grew faster from day three to day five after the initiation of culture. The growth ceased after day five and senescence started at day seven. An OD_{600} value of 0.1 was adequate at the initial stage of culture.

As dosages increased from 2.5 to 13 mL/100 mL, the average PCV in the medium did not increase significantly toward the end of day seven although higher concentration had a faster rate at day three (Fig. 2). This suggests that the seeding dosage at 2.5 mL/100 mL was adequate. The pH values of the medium ranging from 5.5 to 6.2 were found to be adequate for the suspension culture system.

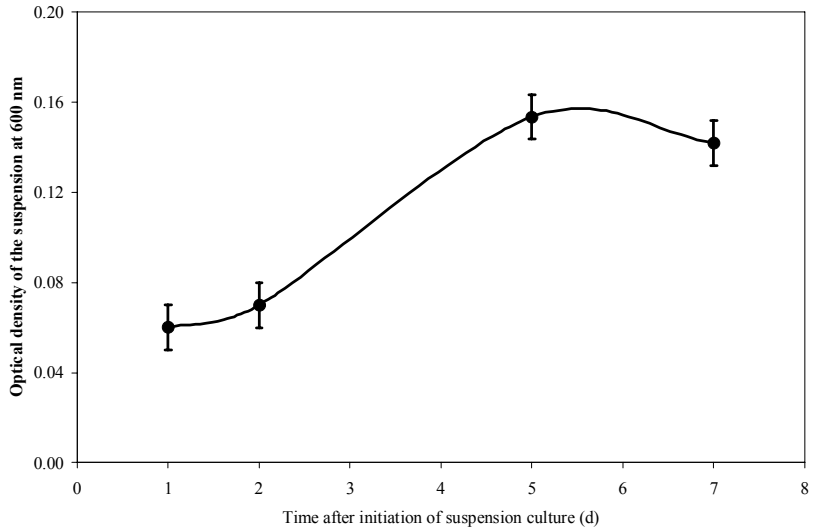

Fig. 1. Volume changes of cell aggregates in suspension during the culture (vertical bars represent standard error).

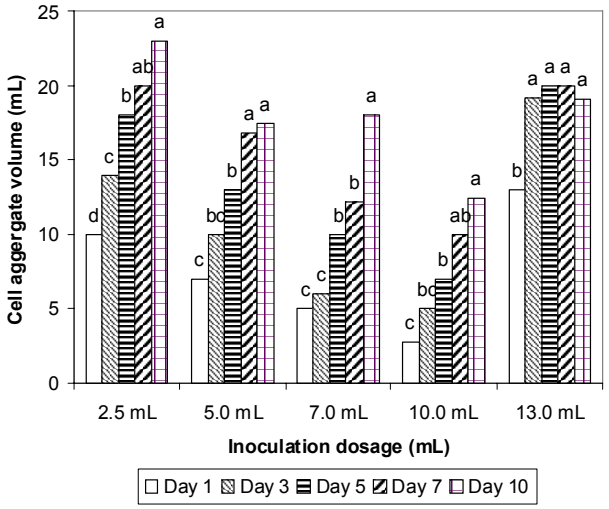

Fig. 2. Influence of initial cultural dosage on the cell aggregate growth in suspension (values with same letters within each dosage are not significantly different at the 0.05level of probability).

Callus induction rate was the greatest on MS_2 media supplemented with Cu^{++} at 2.5 mg L^{-1} (Table 2). At Cu^{++} concentration of 5 mg L^{-1}, callus growth was inhibited and browning increased. Our results were in agreement with the results in tall fescue by Hu et al. (2003). Yang et al. (1999) found that Cu^{++} at higher concentrations

increased callus growth and plant regeneration in barley, wheat, tobacco, and rice plants. It seems that optimum concentration of Cu^{++} is species specific.

The aggregates sieved in the suspension through a 120-㎛ filter appeared to be multi-cell calli containing 1 to15 cells each. These cells had various shapes. At initial stage, most cells had rectangular shape with thick cell wall and large vacuoles. As the culture progressed, the percentage of round cells increased, and eventually, when the suspension was established, most cells were round and oval of uniform sizes and rich in cytoplasm.

Plantlets were generated from the calli after 20 days of culturing on alternative medium. Whole plants were generated after two months of culture with an average rate of 58% (Fig. 3).

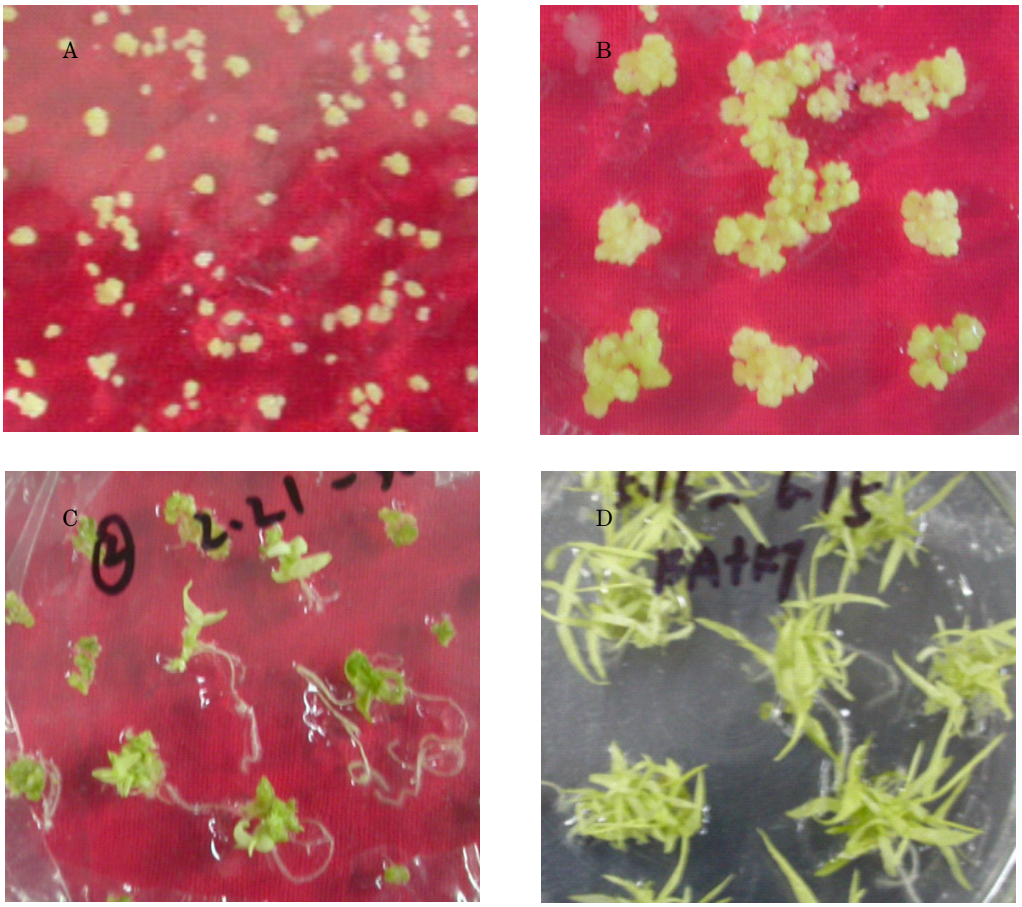

Fig. 3. Aggregates from suspension culture (1-2 mm) cultured on regeneration medium (A), after one mo of culture under light (B), after twomo of culture under light (C), and regenerated plants (D).

Conclusion

MS_2 was the proper medium for callus induction. The Cu^{++}improved the rate of embryo callus induction but at the same time increased the differentiation and browning rate, the balance is at a concentration of 2.5 mg L^{-1}. With the embryonic callus suspension from this study, it was possible to regenerate zoysiagrass plants at a rate of 58%.

국문 요약

한국들잔디는 운동경기장이나 골프 코스 등에 폭넓게 쓰이는 난지형 잔디이다. 많은 품종은 영양체로 번식한다. 이 연구는 배아세포 유도와 식물체 재생을 위한 적정 배양액 개발과 배양조건을 연구하여, 한국잔디의 육종과 번식을 위한 세포배양 체계 확보를 목적으로 하였다. 그 결과 Cu^{++} 2.5mgL^{-1}를 유도 배양액에 첨가하는 것이 캘러스 유도를 위한 최적 농도 조건 이였음을 보여주었다. Sub-culture의 횟수의 증가는 캘러스의 질을 향상시켰다. 세포 부유배양을 위한 최적 용량은 2.5에서 10mL 사이였으며 본 연구에서 58%의 식물체 재생산비율을 보였다.

주요어 : 배, 유전자원, 육종, 잔디

References

1. Al-Kharyi, J.M., F.H. Huang, L.F. Thompson, and J.W. King. 1989. Plant regeneration of zoysiagrass from embryo-derived callus. Crop Sci. 29:1324-1325.
2. Asano, Y. 1989. Somatic embryogenesis and protoplast culture in Japanese lawngrass (*Zoysia japonica*). Plant Cell Rep. 8:141-143.
3. Brede, D. 2000. Turfgrass maintenance reduction handbook: sports, lawns, and golf. Ann Arbor Press, Chelsea, MI.
4. Engelke, M.C., and S. Anderson. 2003. Zoysiagrass. p.271-285. *In* M.E. Casler and R.R. Ducan (eds.) Turfgrass biology, genetics, and breeding. John Wiley & Sons, Inc. Hoboken, NJ.
5. Ge, Y.X., T. Norton, and Z.Y., Wang. 2006. Transgenic zoysiagrass (*Zoysia japonica*) plants obtained by Agrobacterium-mediated transformation. Plant Cell Rep.25:792-798.
6. Hu, Z.H., H.Q. Chen, and G.T. Wu. 2003. Establishment of suspension cell system and high frequency of plant regeneration. Grass Sci. 12:95-99 [*in Chinese*].

7. Inokuma, C., K.Sugiura, N.Imaizumi, and C.Cho. 1998. Transgenic Japanese lawngrass (*Zoysia japonica* Steud) plants regenerated from protoplasts. Plant Cell Rep. 17:334-338.
8. Inokuma, C., K.Sugiura, N.Imaizumi, C.Cho, and S. Kaneko. 1997. Transgenic zoysia (*Zoysia japonica* Steud) plants regenerated from protoplasts. Int. Turfgrass Soc. Res. J. 8:297-303.
9. Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bioassay fortobacco tissue culture. Plant Physiol. 15:473-497.
10. Toyama, K., C.H. Bae, J.G. Kang, Y.P. Lim, T. Adachi, K.Z. Rui, P.S. Song, and H.Y. Lee. 2003. Production of herbicide-tolerant zoysiagrass by agrobacterium mediated transformation. Mol. Cell 16:19-27.
11. Wang, Z., A. Hopkins, and R. Mian. 2001. Forage and turf grass biotechnology. Plant Sci. 20:573-619.
12. Yang, Y.S., Y.Y. Jian, and Y.D. Zheng. 1999. Promoting effect of Cu^{++} in callus induction and plant regeneration. China Rice Science 13:95-98 [*in Chinese*].
13. Yu, S.W., and Y.S. Zhu. 2001. Researches on rapid establishment of embryogenic cell suspension. Journal of Huazhong Agricultural University 20:325-328.