J. Plant Biotechnology (2006) Vol. 8 (1). pp. 21~25

Journal of Plant Biotechnology

In vitro Regeneration of *Phragmites australis* through Embryogenic Cultures

Jeong-Sun Lee[1], Changkyun Kim[2], In Sung Kim[3], Eun Ju Lee[3], Hong-Keun Choi[1*]

[1]Division of Natural Sciences, Ajou University, Suwon 443-749, Republic of Korea; [2]Center for Fungi and Plants Genome Research, FnP Corp., Biovalley #206, Seoul National University, Suwon 441-100, Republic of Korea; [3]School of Biological Sciences, Seoul National University, Seoul 151-742, Republic of Korea

Abstract

***Phragmites australis* (reed) has received much attention as being one of the principle emergent aquatic plants for treating industrial and civil wastewater. Plant regeneration via plant tissue culture in *P. australis* was investigated. Three types of callus were identified from seeds on N6 medium plus 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D). Yellow compact type showed the best redifferentiation, whereas white compact type and yellow friable were not competent to differentiate into plants. Solid medium culture was better than liquid suspension culture for enhancing callus growth when N6 medium supplemented with 4.5 μM 2,4-D was used. Phytagel, as a gelling agent, was superior to agar in plant regeneration on N6 medium, supplemented with 9.4 μM kinetin and 0.54 μM α-naphthaleneacetic acid (NAA). Transfer of the plantlets regenerated from kinetin and NAA-supplemented N6 medium to growth regulator-free MS medium enhanced the further development of the plantlets. Plantlets were subsequently grown to maturity when transferred to potting soil. The regenerated plants exhibited morphologically normal. The system for plant regeneration of *P. australis* enables to propagate elite lines on a large scale for water purification in the ecosystem**

Key words: aquatic plant, callus, plant regeneration, reed, *Phragmites*

* Corresponding author, E-mail: hkchoi@ajou.ac.kr
Received Apr. 1, 2005; Accepted Mar. 9, 2006

Introduction

With the recent occurring of the seriousness of environmental pollution, natural purification method using aquatic plants is gaining power as a method for purifying waters that are heavily polluted. There have been many studies conducted on aquatic vascular plants that have water purification characteristics (Gersberg et al. 1986; Kenneth and Biddlestone 1995; Negri and Hinchman 1996). The aquatic vascular plants with water purification characteristics that elongate the rhizome in the soil and develop the caulis and leaves to be above the water surface removes the water contaminants by directly absorbing and accumulating the nutrient salts, such as nitrogen and phosphorus, and non-biodegradable materials such as heavy metals (Gray 1989; Cooper and Boon 1987). *Phragmites australis* (Cav.) Trin. *ex* Steud. belongs to the family Gramineae is one of the most important and abundant species used in wastewater purification along with *Scirpus validus* L., *Typha angustifolia* L., and *Zizania latifolia* L. (Nichols 1983; Hammer 1996; Choi 2000). In the analysis of the growth rate after exposing a number of aquatic vascular plants to various water pollution sources, we have confirmed that not only the *P. australis* (reed) is more superior in the elongation and vegetative growth compared to *T. angustifolia* (cattail) and *Z. latifolia* (wild rice), but also it absorbs and eliminates over 90% of nitrogen and over 60% of phosphorus.

Aquatic macrophytes propagation via plant tissue culture offers a promising alternative to produce large numbers of plants in a short time throughout the year (Lauzer et al. 2000; Wang et al. 2004). The inclusion of a callus stage during plant regeneration process provides the opportunity for the accumulation of more diversity through somaclonal

variation (Larkin and Scowcroft 1981). Plant regeneration via tissue culture in *P. australis* was first described by Sangwan and Gorenflot (1975), using a stem explant. However, regeneration by organogenesis was sporadic and the authors reported that they found no evidence for somatic embryogenesis. Straub et al. (1988) induced callus from seedling, there was no discussion as to whether or not regenerated plants were derived from somatic embryos. Although plant regeneration from stem or immature inflorescence explant-derived callus cultures of *P. australish* as already been reported (Lauzer et al. 2000; Mathe et al. 2000; Yang et al. 2003), regeneration and massive propagation through somatic embryogenesis from seed callus has not been reported so far. Here we describe the induction of embryogenic callus derived from seed, and establishment of somatic embryogenesis and plant regeneration systems. Accordingly, as part of a natural purification method using plants, we plan to put *P. australis* that is excellent in removing the water contaminants into practical use in areas where the water is polluted through massive propagation using the tissue culture method.

Materials and Methods

Plant materials

Mature seeds of *Phragmites australis* (Cav.) Trin. *ex* Steud. were collected at Nanji Island (E 126° 52' 49", N 37° 34' 56", Seoul, Korea) in 2002. The seeds were surface-sterilized in 2.5% (w/v) sodium hypochlorite solution containing 0.1% (v/v) Tween 20 for 15 min and followed by rinsing three times in sterile distilled water.

Embryogenic callus induction

For callus induction, the seeds were cultured on N6 basal medium (pH 5.8) supplemented with 30 g/L sucrose, 4.5 μM 2,4-D and 2 g/L Phytagel (Lee et al. 1999; Park et al. 2003). The frequency of callus formation on seeds was determined after 30 days of culture. The level of callus proliferation on a solid and in liquid medium was compared after measuring the weight of the callus. In order to investigate the effect the solidifying materials of medium, 8 g/L agar(Sigma Co.) and 2 g/L Phytagel(Sigma Co.) were added to the basal medium and cultivated for approximately 60 days. All data were analyzed using analysis of variance (ANOVA) and means of significant data were separated with Duncan's multiple range test (Duncan 1955) using SAS program (Ver. 6.12, SAS Institute).

Induction of somatic embryo and plant regeneration

For the differentiation of the somatic embryos, the N6 basal medium that included 30 g/L sucrose, 9.4 μM kinetin and 0.54 μM NAA was used. The number of plantlets, length and number of new vagina and roots, and fresh weight were analyzed and compared. The plantlets were transferred into the soil after being cultivated for 4 weeks in basal medium that had not been treated with growth regulators after adding 50 g/L sucrose and 2 g/L Phytagel to MS culture medium. Cultures maintained in the dark were used when inducing the callus from the seeds of *P. australis*. The light conditions for cultures were set at 24 μmol s^{-1} m^{-2} (PAR) at 16 hr photoperiod. The temperature of the cultivating room was about 26℃ and the R.H. of the culture room was maintained at about 60%.

Results and Discussion

Selection of plant materials

Since wild reed plants propagate mainly by spreading rhizomes, it is very hard to meet the need of large amount of reed plants for wastewater purification. So the massive propagation through tissue culture could be a good solution (Wang et al. 2001; Lauzer et al. 2000; Yang et al. 2003). Furthermore it is important to select the superior populations or individuals for improving the efficiency of wastewaster treatment. *Phragmites australis* population used in this study, was selected by test for germination and its capability for absorbing and eliminating the contaminant of other water pollution sources among the land fill sites, the inland wetland, the mine dumps, and the salty wetland (data not shown).

Callus induction

In order to induce the callus from the seed of *P. australis*, N6 basal medium that was added with 4.5 μM 2,4-D was used. Callus induction was observed after 4 weeks of culture. Calluses that formed on the seeds appeared slime even after several subcultures at 4 week intervals. Three types of calluses were identified: (A) yellow, solid callus, (B) white, solid callus, and (C) yellow, friable callus. The frequency of yellow, solid callus formation was 49.3% and the frequencies of white, solid callus formation and yellow, friable callus formation were 40% and 10.7%, respectively. These three types of calluses were transferred to differentiation medium and their fate was observed. There are

Table 1. Effect of methods of culture on the callus growth in *Phragmites australis* after 4 weeks of culture

Methods of culture	Fresh weight (mg/plant)	Callus color[b]
Solid culture[a]	134.8A[c]	Y
Liquid suspension culture	55.4B	Y-B

[a] N6 basal medium containing 30 g/L sucrose and 4.5 μM 2,4-D was used.
[b] Y, yellow; B, brown
[c] Mean separation within columns by Duncan's multiple range test, at 5% level.

various reports that the rate of plant regeneration is related to the callus type. Lee et al. (1999) reported that the plant regeneration occurred relatively highly from white, solid callus than other types of calluses. Likewise, Cho et al. (2003) reported that yellow, friable callus gave rise to higher frequency of plant regeneration. As for *P. australis*, the yellow, solid callus exhibited the highest frequency of plant regeneration in this study. Other types of calluses did not seem competent to regenerate plants. Expecially, friable calluses contained larger, highly vacuolated cells and seemed to be equivalent to typical non-embryogenic callus described in the study of Gramineae (Vasil and Vasil 1984; Straub et al. 1988).

As for lilies, there is a report on reducing the period for subculture of callus by half through liquid suspension culture (Park et al. 1997). As a result of conducting liquid suspension culture in order to find out the difference of culture methods on the callus formation of *P. australis*, under similar conditions, solid culture was better for the fresh weight increase of callus than the liquid suspension culture (Table 1). Although several groups have reported the large-scale propagation of callus through the suspension cultures in *P. australis*, the liquid culture system sometimes leads to problems, such as abnormal embryos and hyperhydricity (Yang et al. 2003).

Somatic embryogenesis and plant regeneration

The solid culture that was made by adding 30 g/L sucrose, 9.4 μM kinetin and 0.54 μM NAA to the N6 basal

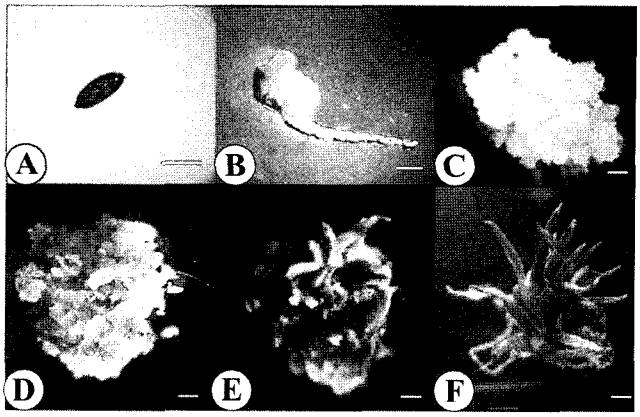

Figure 1. Callus formation and plant regeneration in *Phragmites australis*. (A) Seed; (B-C) callus induced from seed on N6 basal medium with 4.5 μM 2,4-D (B: after 4 weeks in culture, C: after 8 weeks in culture); (D-F) Plantlet development from embryogenic callus cultured on N6 medium with 0.54 μM NAA and 9.4 μM kinetin. Bar=1 mm.

medium appeared adequate for plant regeneration from the callus (Figure 1). The development of somatic embryos depends on the concentration of auxin. Relatively low auxin concentration is helpful for the differentiation of somatic embryos (Straub et al. 1988). When the embryogenic calluses were cultured on N6 medium supplemented with 4.5 μM 2,4-D, they proliferated. When callus was transferred to the medium with relatively low auxin (NAA, 0.54 μM), somatic embryos underwent development.

The number of plantlets differentiated from the callus did not show any significant difference between the different kinds of gelling agents. However, the overall growth and fresh weight was higher in Phytagel than in agar (Sigma Co.) (Table 2). The selection of gelling agent for specific plants is often empirical. One major consideration is the degree of hyperhydricity induced by the different gelling agent. Also, for unknown reasons, some species grow more vigorously on one gelling agent than on another. Apple shoots became hyperhydric on Phytagel whereas walnut shoots grew best on Phytagel and poorly on agar (Pasqualetto et al. 1986; McGranahan et al. 1988).

Regenerated plantlets of *P. australis* were cultured further for about 4 weeks on growth regulator-free MS basal me-

Table 2. Effect of gelling agents on plant regeneration from the callus of *Phragmites australis* after 4 weeks of culture

Gelling agent[a] (g/L)	No. of plantlets	Shoot length (mm)	No. of shoot	Root length (mm)	No. of root	Total fresh weight (mg)
Agar (8)	10.8A[b]	2.6B	3.3B	0.3B	1.0B	9.2B
Phytagel (2)	11.0A	8.6A	5.5A	4.0A	3.0A	41.5A

[a] N6 basal medium containing 30g/L sucrose, 0.54 μM NAA and 9.4 μM Kinetin was used.
[b] Mean separation within columns by Duncan's multiple range test, at 5% level.

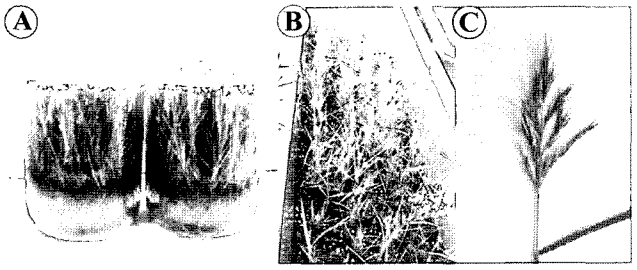

Figure 2. Propagation of large numbers of regenerated plants in *Phragmites australis* (A) Regenerated plants; (B) Transplanted to potting soil; (C) Flowering in transplanted plants.

dium with 50 g/L sucrose. Afterwards, they were transplanted into potting soil. It flowered 9 months after transplanting into the soil (Figure 2). All the plants appeared to be phenotypically normal, and no somaclonal variation was found.

Somatic embryogenesis has been studied extensively in numerous herbaceous species including the family Gramineae (Krishnaraj and Vasil 1995; Vasil 1987). Somatic embryogenesis of Gramineae is usually induced from tissues and organs which contain meristematic and undifferentiated cells such as immature embryos and young inflorescences, and leaves are used as an explant (Ozias-Akins and Vasil 1982; Lauzer et al. 2000). In this study, regeneration through somatic embryogenesis of *P. australis* was observed using mature seeds *in vitro*. Our results are similar to those Lauzer et al. (2000) who showed the presence of bipolar development with a clear root and shoot meristem. Thus, our results confirm the conclusions of Lauzer et al. (2000) that regeneration proceeded through somatic embryogenesis and not by organogenesis.

In order to restore the environment-friendly ecosystem, the need for selecting aquatic vascular plants that can grow well in polluted water environments is increasing (Gray 1989; Hammer 1996; Nichols 1983). As can be seen through this study, somatic embryogenesis induced from the seed of *P. australis* showed that the totipotency of the plant cell was maintained during the development process (Figure 1). Such differentiation totipotency of the somatic cell could be used as a method for massive propagation through *in vitro* culture of individuals with the same genetic characteristics, when elite lines of *P. australis* are selected for wastewater purification.

Acknowledgements

This study is part of the research program on 'Technology for restoring the damaged natural ecosystem' under the project for Developing Next Generation of Key Environmental Technology (No. 051-041-006). We thank two anonymous reviewers for helpful comments and Dr. Liu, JR (KRIBB) for his critical revision on an earlier version of this manuscript. We also wish to express our appreciation for the cooperation provided by NAKOS Engineering Co., a partner in this project.

References

Cho MC, Juang UD, Park SG, Park Y (2003) Regeneration and acclimatization of plants derived from anther cultures in carrot (*Daucus carota* L.). Kor J Plant Biotechnology 30: 47-52

Choi H.-K. (2000) Aquatic Vascular Plants. A series of Korean Plants 5. Jungheng Publishers, Seoul, pp 9-13

Cooper PF, Boon AG (1987) The use of *Phragmites* for wastewater treatment by the root zone method: The UK approach. In: Reddy KR, Smith WH (eds), Aquatic Plants for Water Treatment and Resource Recovery, Mangnolia Pub Inc, Orlando, Florida, pp 153-174

Duncan DB (1955) Multiple range and multiple *F* test. Biometrics 11: 1-42

Gersberg RM, Elkins BV, Lyons RS, Goldman CR (1986) Role of aquatic plants in wastewater treatment by artificial wetlands. Water Research 20: 363-368

Gray NF (1989) Biology of Wastewater Treatment. Oxford Univ Press, pp 828

Hammer DA (1996) Creating Freshwater Wetlands. Lewis Publisher, New York, pp 406

Kenneth RG, Biddlestone AJ (1995) Engineered reed-bed systems for wastewater treatment. Trend Biotechnol 13: 248-252

Krishnaraj S, Vasil IK (1995) Somatic embryogenesis in herbaceous monocots. In: Thorpe TA (ed) *In Vitro* Embryogenesis in Plant. Kluwer Academic Press, Dordrecht, pp 417-470

Larkin PJ, Scowcroft WR (1981) Somaclonal variation-a novel source for variability from cell culture for plant improvement. Theor Appl Genet 60: 197-214

Lauzer D, Dallaire S, Vincent G (2000) *In vitro* propagation of reed grass by somatic embryogenesis. Plant Cell Tiss Org Cult 60: 229-234

Lee HY, Jeon JO, No JG, Park HG (1999) Effects of several factors on callus induction in anther culture of cherry tomato. J Kor Soc Hort Sci 40: 537-540

Lee S, Jeon JS, Jung KH, An GH (1999) Binary vectors for efficient transformation of rice. J Plant Biol 42: 310-316

Mathe C, Hamvas MM, Grigorszky I, Vasas G, Molnar E, Power JB, Davey MR, Borbely G (2000) Plant regeneration from embryogenic cultures of *Phragmites australis* (Cav.) Trin. *ex* Steud. Plant Cell Tiss Org Cult 63: 81-84

McGranahan G, Leslie CA, Driver JA (1988) *In vitro* propagation of mature Persian walnut cultivars, HortSci 53: 63-72

Negri MC, Hinchman RR (1996) Plants that remove contaminants from the environment. Laboratory Medicine

27: 36-40

Nichols DS (1983) Capacity of natural wetlands to remove nutrients from wastewater. Res Jour WPCF 55: 495-505

Ozias-Akins P, Vasil IK (1982) Plant regeneration from cultured immature embryos and inflorescences of *Triticum aestivum* L. (wheat): Evidence for somatic embryogenesis. Protoplasma 110: 95-105

Park MC, Shin JS, Kim NR, Cho HJ, Cho HJ, Park SH, An KS, Lee SC, An GH (2003) High-frequency agrobacterium-mediated genetic transformation of Tongil rice varieties. J Plant Biol 46: 23-30

Park SY, Kim SD, Sin SK, Lee CH, Paek KY (1997) Several factors on bulblets regeneration from callus culture in *Lilium longiflorum* 'Gelia'. Kor J Plant Tiss Cult 24: 183-188

Pasqualetto PL, Zimmerman RH, Fordham I (1986) Gelling agent and growth regulator effects on shoot vitrification of 'Gala' apple *in vitro*. J Am Soc Hort Sci 111: 976-980

Sangwan RS, Gorenflot R (1975) *In vitro* culture of *Phragmites* tissue: Callus formation, organ differentiation and cell suspension culture. Z Pflanzenphysiol Bd 75 S: 256-269

Straub PF, Decker DM, Gallagher JL (1988) Tissue culture and long-term regeneration of *Phragmites australis* (Cav.) Trin. ex Steud. Plant Cell Tiss Org Cult 15: 73-78

Vasil IK (1987) Developing cell and tissue culture systems for the improvement of cereal and grass crops. J Plant Physiol 128: 193-218

Vasil V, Vasil IK (1984) Induction and maintenance of embryogenic callus cultures of Gramineae. In: Vasil IK (ed), Cell Culture and Somatic Cell Genetics of Plants. Academic Press, New York, pp 36-42

Wang J, Seliskar DM, Gallagher JL (2004) Plant regeneration via somatic embryogenesis in the brackish wetland monocot *Scirpus rubustus*. Aquatic Botany 79: 163-174

Wang W, Cui SX, Zhang CL (2001) Plant regeneration from embryogenic suspension cultures of dune reed. Plant Cell Tiss Org Cult 67: 11-17

Yang YG, Guo YM, Guo Y, Guo ZC, Lin JX (2003) Regeneration and large-scale propagation of *Phragmites communis* through somatic embryogenesis. Plant Cell Tiss Org Cult 75: 287-290