Note Plant Biotechnology, 19 (1), 63-68 (2002)

A Simple and Efficient Culture of Leaflets for Plant Regeneration in Guineagrass (*Panicum maximum*)

Lanzhuang CHEN[1]*, Reiji OKABE[2], Liming GUAN[1] and Taiji ADACHI[3]

[1] *Gene Research Center,* [2] *Faculty of Agriculture, Miyazaki University, 1-1 Gakuen Kibanadai Nishi, Miyazaki 889-2192, Japan;* [3] *Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka 599-8531, Japan*

**Corresponding author* E-mail address: chenlz@cc.miyazaki-u.ac.jp

Received 31 August 2001; accepted 30 October 2001

Abstract

A simple and efficient culture for plant regeneration was established from the leaflet tissue of *Panicum maximum*. The vegetative leaflets were excised from terminal tillers arising at the end of stolons and cultured for callus formation on Murashige and Skoog (MS) medium supplemented with 2,4-dichlorophenoxyacetic acid. After 30 days of culture, compact callus formed. And after 60 days, white embryogenic calli were induced with wave-like-shapes. When the embryogenic calli were transferred onto MS medium supplemented with kinetin and giberellic acid for regeneration, they developed and formed multiple shoots. The rooted plants were obtained within 4 months. All of the regenerants were successfully grown to maturity.

Keywords: Apomictic grass, Leaflet culture, *Panicum maximum* Jacq., Plant regeneration, Scanning electron microscopy, Somatic embryogenesis.

Abbreviations

GA3, Giberellic acid; Kin, Kinetin; MS medium, Murashige and Skoog medium; 2,4-D, 2,4-dichlorophenoxyacetic acid.

Recovery systems for regenerable callus and suspension cultures have been established for many Gramineae species (Lazzeri and Lorz, 1990; Akashi and Adachi, 1992; Vasil *et al.*, 1992). These systems have been used as a powerful tool in plant cell and molecular biology. Of particular note is the fact that plant regeneration can be achieved by employing these systems, meaning they can be used not only in micropropagation but also in producing transgenic plants by the introduction of foreign DNA. We have been successful in cloning a candidate apomixis-specific gene (ASG-1) using facultative apomictic guineagrass (*Panicum maximum*) (Chen *et al.*, 1999a, b). To identify the gene's functions we are interested in establishing a system of somatic embryogenesis and plant regeneration from leaflets of guineagrass. In particular, sexual guineagrass materials are important as they can confirm that the introduced gene is being expressed (Hanna and Bashow, 1987). The culture system of apomictic guineagrass provides extra expression after introduction of the gene. The objectives of this study were to establish a simple and efficient plant regeneration system via somatic embryogenesis from leaflet tissue that can be obtained in any season and at any time and then be used to make transgenic plants.

Two varieties, Gatton and Petrie, and 4 accessions of guineagrass (*Panicum maximum* Jacq.) collected from Tanzania, Kenya and Japan, were kindly provided by the Laboratory of Plant Genetic Resources, Kyushu National Agricultural Experiment Station (Japan) and used in this study. The plants were potted, grown in a greenhouse and used as culture materials for explants. Leaflet tissues were taken from terminal tillers arising at the end of stolons (**Fig. 1A**), or from those parts of the meristem where spikes had not emerged. About 5 cm of leaflets were isolated by trimming the leaves and excising the parts of the leaf-stem tissue 1 cm above the root base or the last node which contains the basal meristem and basal leaf tissue. The outer 3 or 4 leaves of the tissue were removed. The remaining leaflet tissues of about 2-3 cm were cut into 5 mm lengths and washed in streaming tap water for 30 min. The explants were transferred to 70% ethanol for 1 min., sterilized in 0.15% sodium hypochlorite solution (Antiformin) for 15 min. and rinsed

in sterile distilled water three times for 5 min. each. The prepared explants were then placed on Murashige and Skoog (1962) medium (MS) for callus formation (**Fig. 1B, C**).

The effects of hormones on callus formation were tested on the MS medium supplemented with 5 and 10 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 30 g l^{-1} sucrose, 2.5 g l^{-1} Gellan Gum (Wako Pure Chemical Industries, Ltd. Japan) and the cultures were incubated in the dark at 25 ℃ . Calli were subcultured every 3 weeks and the rates of callus formation were observed 30 days after culturing. Embryogenic calli were initiated from yellowish-calli-derived calli and their rates were observed on 60 days after culturing. Embryogenic calli were prepared for SEM according to Chen *et al.* (2001). The selected embryogenic calli were then transferred onto MS medium supplemented with 1.0 mg l^{-1} kinetin (Kin) and 1.0 mg l^{-1} giberellic acid (GA3) for plant regeneration at 25 ℃ and in lighted conditions (photon flux density = 33.3 $\mu mol^{-2}s^{-1}$, 16 h). The rates of shoot regeneration were observed after 12 weeks. The shoots formed were acclimated according to Chen *et al.* (2001).

By taking vegetative organs, like leaflets, embryogenic callus induction is a very practical and useful method for plant regeneration in species of *Gramineae* because previously only mature and immature reproductive organs had been successfully used to regenerate calli (Metzinger *et al.*, 1987; Vasil, 1988; Akashi and Adachi, 1992). Our first attempts to introduce embryogenic callus from young leaves,

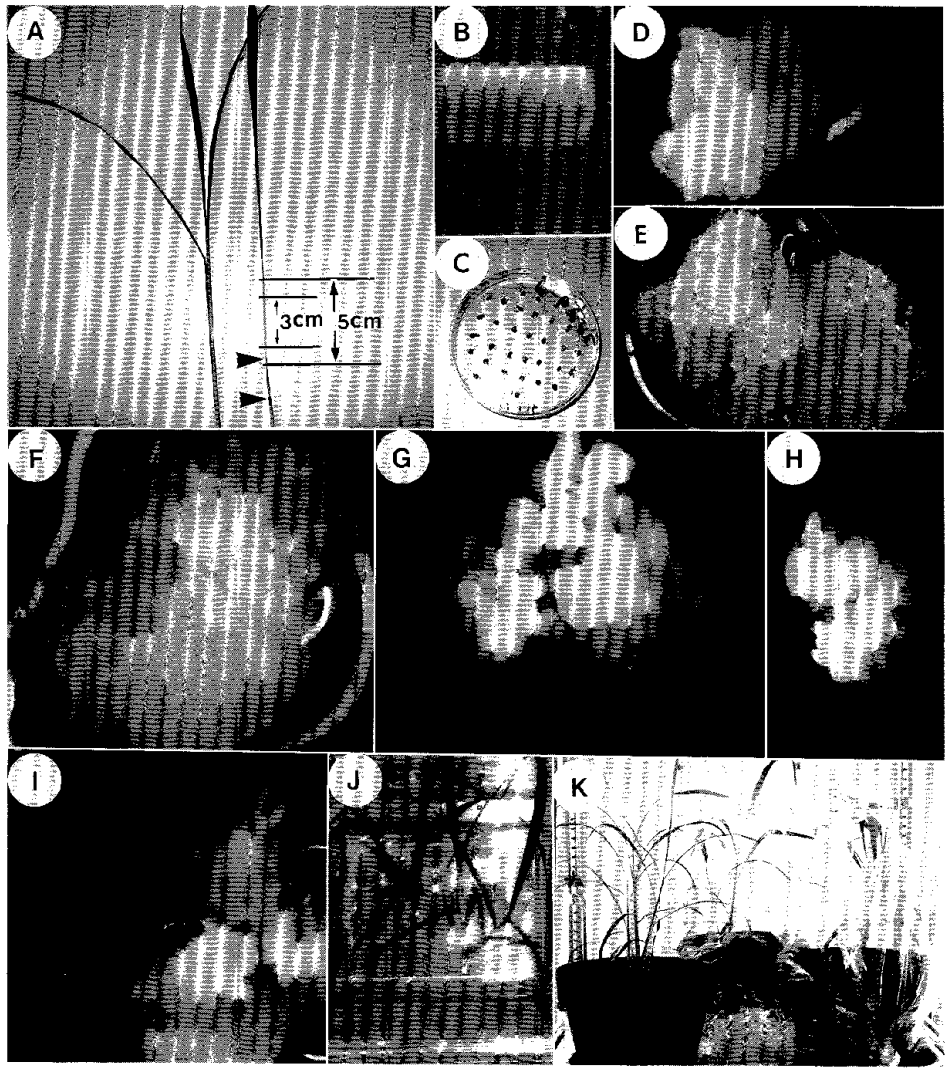

Fig. 1 Somatic embryogenesis and plant regeneration from leaflets of *Panicum maximum*. (A) Leaflet sampling method. (B) and (C) Five mm length leaflet explant placed on MS medium for callus formation. (D) Swelling of explant after 5 days of culture. (E) Callus formation from both central parts of the explant after culturing for 14 days. (F) Formation of compact calli with yellowish and white colors (type I) after culturing for 30 days. (G) Wave-like shaped calli formation after culturing for 60 days. (H) Somatic embryos cut and placed on regeneration medium. (I) Multiple shoots sprouting from a somatic embryo. (J) Plant regeneration with roots on hormone-free medium. (K) Plant acclimation.

stems and roots were unsuccessful. Consequently, the calli were initiated from leaflets. After 5 days of culture, swelling of the tissue was the first visible sign of morphology (**Fig. 1D**). As the cultures progressed, calli were formed 14 days from both central parts of the tissue. The calli were a white-yellowish color and soft (**Fig. 1E**). Compact calli with a yellowish color were formed around the tissue after 30 days of culture (**Fig. 1F**). In this case, the external tissue of the leaflet was usually not able to induce callus formation and subsequently became brown in color. The compact calli were called Type I callus, from which the embryogenic calli could easily emerge. After 60 days of culture, calli with a wave-like shape and white color were formed on the surface of Type I calli (**Fig. 1G**). To identify the ultrastructures of the Type I and wave-like shaped calli, SEM was performed. The callus of Type I (**Fig. 1F, Fig. 2A**) showed 2 kinds of structures, a smaller tube shape (**Fig. 2B**) and a bigger and thicker tube shape (**Fig. 2C**). The white wave-like shaped callus (**Fig. 1G, Fig. 2D**) had somatic embryos with multiple plumule formation (**Fig. 2E**) and extended plumule sprouted from the coleoptile (**Fig. 2F**). These structures were similar to those of the somatic embryos previously reported in *Panicum maximum* Jacq. (Lu and Vasil, 1985), Paspalum notatum Flugge (Marousky and West, 1990) and *Paspalum dilatatum* Poir (Akashi and Adachi, 1992). **Table 1** shows the rates of calli and embryogenic calli regeneration from 6 genotypes after 30 and 60 days of culture at different 2,4-D concentrations. There was no significant correlation between the 2,4-D concentrations and callus formation, the calli with higher rates (34.5-88.6%) were produced using both types of media. However, the rates of embryogenic calli formation among 6 genotypes were higher at 5 mg l^{-1} than that at 10 mg l^{-1} 2,4-D, except N68/96-8-o-7 (S). The highest rate of embryogenic callus formation from leaflets was obtained on MS medium supplemented with 5 mg l^{-1} 2,4-D. The embryogenic callus culture system has been maintained for 16 months, using Type I callus culture on MS medium supplemented with 5 mg l^{-1} 2,4-D every 2 weeks. Embryogenic callus formation from vegetative organs was first reported

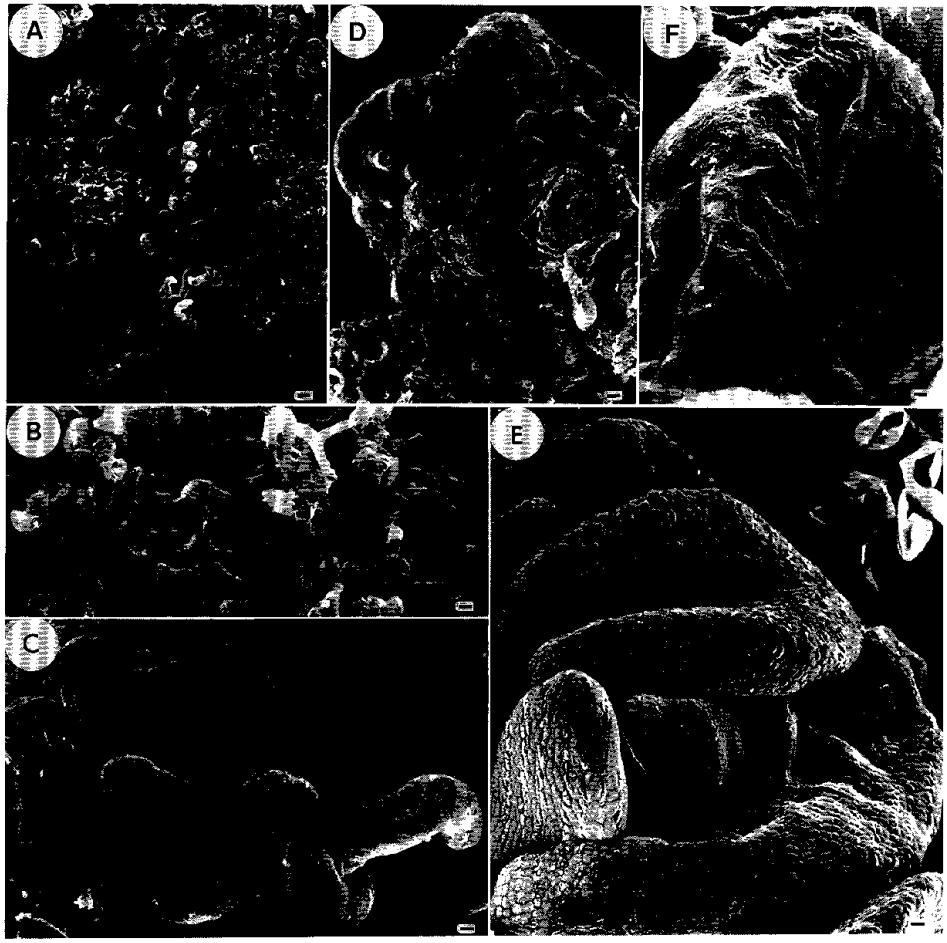

Fig. 2 Scanning electron microscopy of type I calli. (A) Whole view of the calli. (B) Smaller tube-like shaped structure. (C) Bigger tube-shaped structure. (D) Wave-shaped callus. (E) Formation of multiple plumule. (F) Sprouting of plumule. Bars = 89, 12, 12, 97, 40 and 30 μm in (A), (B), (C), (D), (E) and (F), respectively.

Table 1 The effects of hormones on embryogenic callus formation from leaflets of guineagrass[1)]

Guineagrass accessions	2, -4-D (mgl^{-1})	No. explants cultured	No. explants forming callus (%)[2)]	No. explants forming embryogenic callus (%)[3)]
N68/96-o-11 (A)	5	167	148 (88.6)	10 (6.0)
N68/96-o-11 (A)	10	160	94 (58.8)	8 (5.0)
Gatton (A)	5	91	41 (45.1)	8 (8.8)
Gatton (A)	10	177	96 (54.2)	2 (1.2)
Petrie (A)	5	95	59 (62.1)	14 (14.8)
Petrie (A)	10	106	78 (73.6)	2 (1.8)
N68/96-8 (S)	5	178	96 (53.9)	4 (2.2)
N68/96-8 (S)	10	110	38 (34.5)	0 (0)
N68/96-8-o-5 (S)	5	110	70 (63.6)	0 (0)
N68/96-8-o-5 (S)	10	104	74 (71.2)	14 (13.5)
N68/96-8-o-7 (S)	5	157	90 (57.3)	0 (0)
N68/96-8-o-7 (S)	10	105	85 (76.2)	0 (0)

[1)](A)=Apomictic guineagrass; (S)=Sexual guieagrass. [2)]The number of explants forming callus was observed at 30 days after culture. [3)]The number of explants forming embryogenic callus was observed at 60 days after culture.

Table 2 The effects of hormones on plant regeneration from embryogenic calli of guineagrass[1)]

Guineagrass	Kin (mgl^{-1})	GA3 (mgl^{-1})	No. embryogenic calli cultured	No. calli forming shoots (%)[2)]	No. shoots per callus
N68/96-8-o-11 (A)	0	0	18	0 (0)	0
N68/96-8-o-11 (A)	1	1	17	4 (23.5)	3.5
Petire (A)	0	0	14	0 (0)	0
Petire (A)	1	1	14	3 (21.4)	3
N68/96-8 (S)	0	0	4	0 (0)	0
N68/96-8 (S)	1	1	4	1 (25.0)	4
N68/96-8-o-5 (S)	0	0	14	0 (0)	0
N68/96-8-o-5 (S)	1	1	14	4 (30.4)	3.5

[1)](A)=Apomictifc guineagrass;(S)=Sexual guineagrass. [2)]The number of calli forming shoots was observed at 12th week of culture.

from shoot-leaf explants in bahiagrass (*Paspalum notatum* Flugge L.) by Shatters *et al.* (1994). This report was first to indicate the possibility of embryogenic callus formation from leaflets in guineagrass, even though the rate of embryogenic callus formation was not particular high (14.8%).

Compact and white-colored calli with somatic embryos were selected and propagated in the same medium for two to three rounds of subculture every two weeks. Subsequently, the somatic embryos cut from the calli were transferred onto MS medium supplemented with 1.0 mgl^{-1} Kin and 1.0 mgl^{-1} GA3 for germination (**Fig. 1H**). The embryos began to germinate with the plumule and coleoptile structures only becoming apparent 10 days after transfer (**Fig. 1I**). Usually, germinated leafy structures with anthocyanin pigmentation were observed. This phenomenon is similar to that of *Paspalum notatum* (Chen *et al.*, 2001) and immature inflorescences of *Paspalum dilatatum* (Akashi and Adachi, 1992). When these shoots were excised from the calli and transferred onto hormone-free MS medium, the plantlets and roots were developed (**Fig. 1J**). After acclimation described in M & M, regenerated plants had typical morphology and had a 100% survival rate (**Fig. 1K**). **Table 2** shows the rates of shoot regeneration from somatic embryos on germination medium after 30 days. The rates of shoot regeneration ranged from 23.5 to 30.4% and indicated a high efficiency in the 4 genotypes with no clear differences among them. Albino shoots were not observed. The number of shoots per callus ranged from 3.5 to 4.0 among the 4 genotypes. As MS hormone-free medium gave a good result for ger-

mination in bahiagrass (Chen *et al.*, 2001), the embryogenic calli were also cultured on the same medium. However, the embryos on hormone-free MS medium in guineagrass failed to germinate. This may indicate differences in the level and types of hormones in bahiagrass and guineagrass.

In this study we demonstrate the possibility for somatic embryogenesis and plant regeneration from leaflets in *Panicum maximum*. In forage grasses somatic embryogenesis is usually obtained from mature and immature reproductive tissues at high concentrations of auxin (Zimny and Lorz, 1989; Akashi and Adachi, 1992). The current study agrees with previous studies on the relationship between auxin concentration and callus formation. However, for the 5 genotypes that formed embryogenic calli, 4 genotypes gave higher rates of formation with 5 mg l^{-1} of 2,4-D and one gave a higher rate with 10 mg l^{-1} of 2,4-D (**Table 1**). To regenerate plants from somatic embryos, many researchers have lowered or entirely omitted 2,4-D or other auxins (Vasil and Vasil, 1984; Ahn *et al.*, 1985; George and Eapea, 1990), but they obtained fewer regenerated plants. In our experiments, adding Kin and GA3 for regeneration results in a higher germination of somatic embryos, which agrees with that of immature inflorescences in *Paspalum dilatatum* (Akashi and Adachi, 1992).

Following to the procedure for the plant regeneration system established in this study, complete plants via somatic embryogenesis were achieved from leaflets within 4 months. The availability of field-grown immature inflorescences and immature embryos as experimental materials for inducing embryogenic callus is restricted to a very short season of the year (Akashi and Adachi, 1992). However, using the protocol presented here, experimental material is available at any time. That is, the leaflets used as materials for plant regeneration allow culturing throughout the year, as leaflets can be collected in any season and at any time, unlike some reproductive tissues such as immature inflorescence, immature and/or mature embryo or seeds. Additionally, the recovery of chemical- or radiation-induced mutations or genetically transformed plants should be possible using this technique since somatic embryos are generally believed to arise from single cells (Haccius, 1978). Therefore, this system of rapidly regenerating plants from guineagrass leaflet culture and subcultures of embryogenic calli can be used in genetic engineering experiments, especially for developing transgenic plants. Attempts to transform an apomixis-specific gene (ASG-1) isolated from facultative apomictic guineagrass (Chen *et al.*, 1999a, b) into sexual guineagrass calli are currently in progress.

Acknowledgement

This study was partly supported by the Grant-in-Aid for Scientific Research (C) of The Ministry of Education, Science, Sports and Culture of Japan, No. 10660010.

References

Ahn, B. J., Huang, H. F., King, J. W., 1985. Plant regeneration through somatic embryogenesis in common bermudagrass tissue culture. Crop Sci., **25**: 1107-1109.

Akashi, R., Adachi, T., 1992. Somatic embryogenesis and plant regeneration from cultured immature inflorescences of apomictic dallisgrass (*Paspalum dilatatum Poir.*). Plant Sci., **82**: 213-218.

Chen, L. Z., Anami, E., Guan, L. M., Adachi, T., 2001. Somatic embryogenesis and plant regeneration from leaflets of "Nanou" bahiagrass. Plant Biotechnol., **18**: 119-123.

Chen, L. Z., Guan, L. M., Miyazaki, C., Kojima, A., Saito, A., Adachi, T., 1999a. Cloning and characterization of a cDNA expressed at aposporous embryo sac initial cell appearance stage in Guineagrass (*Panicum maximum* Jacq.). Apomixis Newsletter, **11**: 32-34.

Chen, L. Z., Guan, L. M., Miyazaki, C., Kojima, A., Saito, A., Adachi, T., 1999b. Isolation and characterization of a gene expressed during early embryo sac development in apomictic guinea grass (*Panicum maximum*). J. Plant Physiol., **154**: 55-62.

George, J., Eapen, S., 1990. High frequency plant-regeneration through direct shoot development and somatic embryogenesis from immature inflorescence culture of finger millet (*Eleusine coracana* Gaertn). Euphytica, **48**: 269-274.

Haccius, B., 1978. Question of unicellular origin of nonzygotic embryos in callus cultures. Phytomorphology, **28**: 74-81.

Hanna, W. W., Bashow, E. C., 1987. Apomixis: its identification and use in plant breeding. Crop Sci., **27**: 1136-1139.

Lazzeri, P. A., Lorz, H., 1990. Regenerable suspension and protoplast cultures of barley and stable transformation via DNA uptake into protoplasts. In: Lycett, G. W., Grieson, D. (Eds.): Genetic engineering in crop plants, pp. 231-238. Butterworths, London.

Lu, C., Vasil, I. K., 1985. Histology of somatic embryogenesis in *Panicum maximum* (guineagrass). Amer. J. Bot., **72**: 1908-1913.

Marousky, F. J., West, S. H., 1990. Somatic embryogenesis and plant regeneration from cultured mature caryopses of bahiagrass (*Paspalum notatum* Flugge). Plant Cell, Tissue Organ Cult., **20**: 125-129.

Metzinger, B. D., Taliaferro, C. M., Johnson, B. B., Mitchell, Jr. E. D., 1987. *In vitro* regeneration of apomictic bluestem grasses. Plant Cell, Tissue Organ Cult., **10**: 31-38.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture.

Physiol. Plant., **15**: 473–479.

Shatters, R. G., Wheeler, R. A., West, S. H., 1994. Somatic embryogenesis and plant regeneration from callus cultures of "Tifton 9" bahiagrass. Crop Sci., **34**: 1378–1384.

Vasil, V., Castillo, A. M., Fromm, M. E., Vasil, I. K., 1992. Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Biotechnology, **10**: 667–674.

Vasil, I. K., Vasil, V., 1984. Regeneration in cereal and other grass species. In: Vasil, I. K. (Ed.): Cell Culture and Somatic Genetics of Plants, pp. 121–150. Academic Press, Orlando.

Zimny, J., Lorz, H., 1989. High frequency of somatic embryogenesis and plant regeneration of rye (*Secale cereale* L.). Plant Breed., **102**: 89–100.