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Establishment of Embryogenic Suspension Culture Derived from Leaflets of Sexual Bahiagrass (*Paspalum notatum*) with Regeneration Ability in Long Term

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Abstract

To establish a simple and constant suspension culture system for gene manipulation, the leaflets of "C1" sexual bahiagrass line were used for embryogenic callus formation on Murashige and Skoog's medium (MS) supplemented with 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). The obtained embryogenic calli were broken into pieces for suspension culture in N6 liquid medium (Chu *et al.*, 1975) supplemented with 1 mg l^{-1} 2,4-D. After 2 weeks of cultures two kinds of bigger and smaller calli were obtained. Another 2 weeks later, the bigger ($\geqq$ 4 mm diameter) became brown color and vacuolated, and the smaller ($\leqq$ 2 mm diameter) showed uniform size and dense cytoplasm. The smaller calli were then selected and moved onto the solid medium for regeneration. When the cultures were continued, shoots and plants were regenerated. The abilities of shoot regeneration from suspension culture could be kept at least for 12 months. This study provides a simple and constant plant regeneration system available for making transgenic apomictic plants based on embryogenic suspension culture derived from leaflets of sexual bahiagrass.

Abbreviations

MS, Murashige and Skoog's (1962) medium; Kin, Kinetin; GA3, Giberellic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; SEM, scanning electron microscopy.

Introduction

Suspension culture system with somatic embryogenesis and plant regeneration was first reported in carrot in 1950s. After that many reports on this system appeared mainly in dicots. Though some reports on monocots appeared, like rice, efficiency of regeneration was not enough for practice of gene and cell manipulation. Therefore, it is important to fit today's requirement to establish an efficient and easily operating suspension culture system. In addition, somatic embryogenesis from suspension cultures is easily controlled by environmental conditions, so it is also considered as an excellent experimental system for physiological research of embryogenesis.

The cultures of embryogenic calli have previously been reported in 'Pensacola' bahiagrass. Marousky and West (1990) isolated regenerable calli from seed caryopses and Bienik (1989) demonstrated preparation of regenerable calli from young leaf tissue and immature inflorescence. Later, Shatters *et al.* (1994) reported regenerable calli from leaf sections of 'Tifton'. Recently, Chen *et al.* (2001) isolated regenerable embryogenic calli from leaflets of 'Nanou' apomictic bahiagrass (*Paspalum notatum*). We have obtained successfully apomixis-specific candidate genes, ASG-1 and ASG-2, stage-specific genes of aposporous initial cell appearance from *Panicum maximum* (Chen *et al.*, 1999a, b). As part of our interest in testing whether the genes work in relation to apomixis action or not, a sexual plant regeneration system is essential to be used as foreign gene acceptor. The objects of this study are to determine whether or not regenerable calli could be isolated from suspension culture derived from leaflets of sexual bahiagrass, and to develop the methods for the maintenance of regenerable calli.

Materials and methods

Sexual bahiagrass trace (*Paspalum notatum* Flugge) "C1" was used as experimental materials. "C1" is a diploid (2n = 20) selected as intermediate parental line and kindly provided from Osumi breeding branch, Kagoshima Prefecture Agricultural Experiment Station, Japan. Leaflet tissues of plants grown in greenhouse were sampled according to the manner of Chen *et al.* (2001). Briefly, leaflets were taken from terminal tillers arising at the end of stolons (**Fig. 1**). Two to 3 cm of the leaflets were isolated by trimming the leaves and the parts of the leaf-stem tissue 1 cm above the root base which contains the basal meristem and basal leaf tissue were excised. The outer four to five leaves were removed. The remaining leaflets were cut into 5-mm sections and sterilized in 0.15% sodium hypochlorite solution (Antiformin) for 15 min. The explants were then placed on MS medium supplemented with 2 mg l^{-1} 2,4-D, 30 g l^{-1} sucrose, 2.5 g l^{-1} Gellan Gum (Wako Pure Chemical Industries, Ltd. Japan) for callus induction at 25 ℃ in dark.

Calli were subcultured every 3 weeks and the rates of callus formation were observed at the 3rd week after the culture. Embryogenic calli were initiated from the calli derived from yellowish color calli and the rates were observed on the 9th week. The selected embryogenic calli were crushed with a spoon, sieved through a stainless mesh basket (ϕ 1 mm) and cultured in N6 liquid medium (Chu *et al.*, 1975) with 1 mg l^{-1} 2,4-D and 30 g l^{-1} sucrose. Eight to 10 weeks were needed for the establishment of suspension cultures after calli initiation from leaflets. Cultures were incubated in a gyratory shaker (80-90 rev./min.) at 25 ℃ in the dark and subcultured at intervals of 2 weeks for 2 times, and then, 1 week.

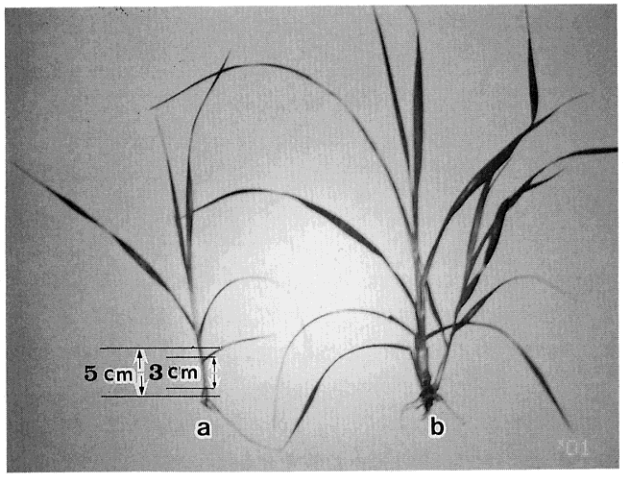

Fig. 1 The materials used as explants for callus production and their excision. a. Tiller; b. Mother stock. For details referring "M & M".

Propagated calli from suspension cultures were moved onto MS medium with 0.1 mg l^{-1} Kin and 1 mg l^{-1} GA3 solidified with 0.2% Gellan Gum for regeneration. Cultures were incubated at 25 ℃ under light condition (photon flux density = 33.3 μmol $m^{-2} \cdot s^{-1}$, 16 h).

Shoots were regenerated from the embryogenic calli. They were removed from the cultures and rooted on phytohormone-free MS medium. The rooted plants were potted in washed sand and covered with plastic bags to keep full humidity at 25 ℃ under light condition (photon flux density = 33.3 μmol $m^{-2} \cdot s^{-1}$, 16 h). As the plants grew vigor, the bags were poked by chopsticks to allow the air enter inside bags until the plants are self-supported. Preparation of embryogenic calli for SEM was carried out according to a previous report (Chen *et al.*, 2001).

Results and discussion

Calli formation and embryogenic suspension culture

When the explants of leaflets were cultured on medium, calli were formed a week later (**Fig. 2A**). Calli appeared not only from the center of the explant but also the surface of the outer tissue. It seemed that the calli emerge from the whole tissue. At 3 weeks of the cultures, the typical watery, yellowish color calli covered the whole tissue (**Fig. 2B**). The rates of callus formation were 25%, which is similar to that of *Paspalum notatum* with direct embryogenesis previously reported by Chen *et al.* (2001). After another 3 and 6 weeks two kinds of calli, nonembryogenic and embryogenic were formed (**Fig. 2C, D**), respectively. As indicated in previous report (Chen *et al.*, 2001), the characteristics of nonembryogenic calli were yellowish-white color, opaque and soft, which were same to that of bahiagrass callus cultures of Shatters *et al.* (1994). And they could not differentiate into embryogenic calli after the cultures were continued even. For the embryogenic calli, they showed white-yellowish color, nodular, and scalloped shapes. After continued culture the yellowish color was gradually lost. The rate of embryogenic calli formation was about 30% at 9th week of the culture. In order to distinguish the structure of somatic embryos, the callus of **Fig. 2D** was observed with SEM. The border with scallop-shaped scutellum (SC) and root-shaped tissues (**Fig. 3A**) was very clear between embryogenic (e) and nonembryogenic parts (n). The somatic embryos were observed with coleoptile (CO) and emerged plumule (**Fig. 3B, C**). The structures of somatic embryos were similar to

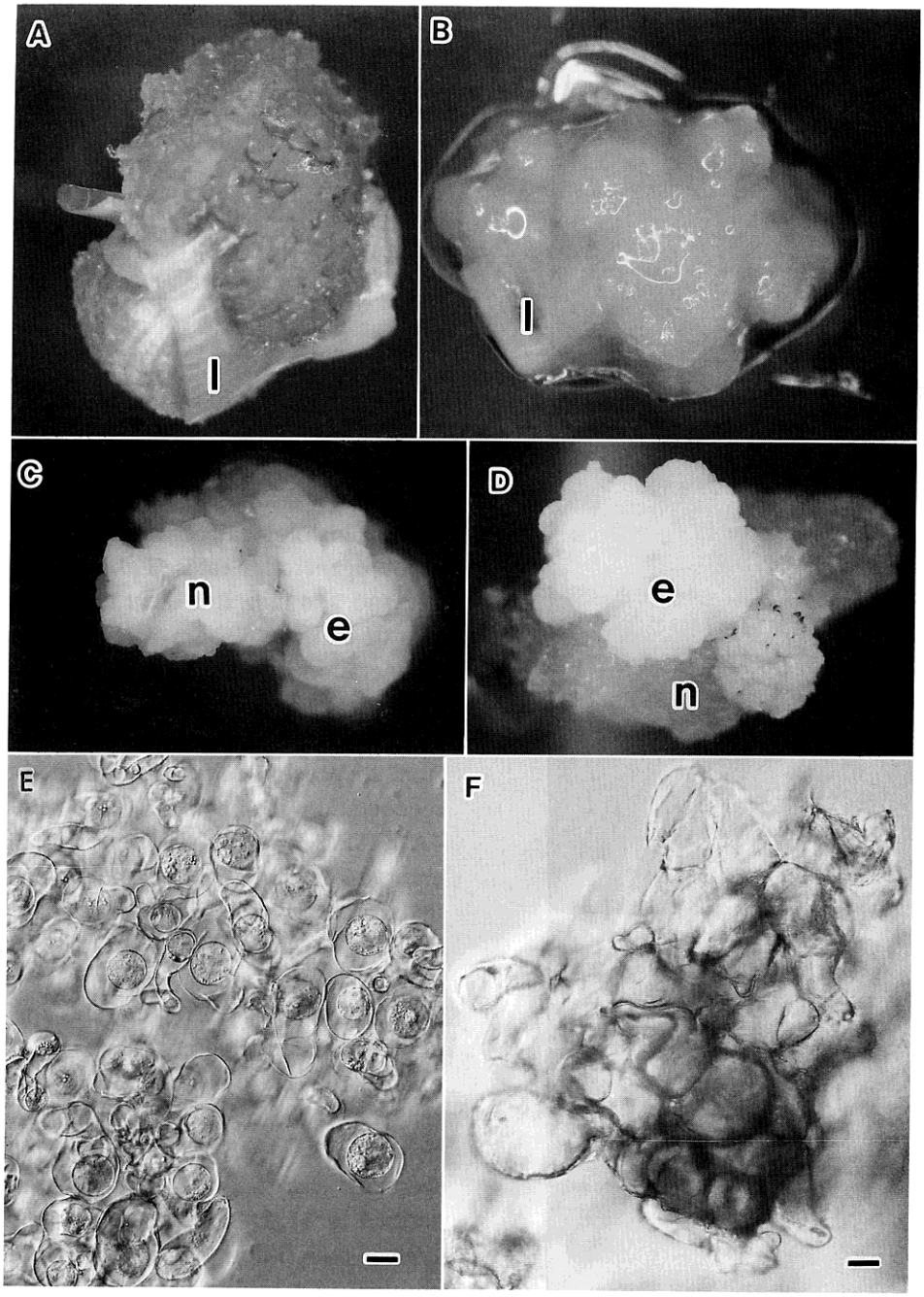

Fig. 2 Embryogenic calli formation from leaflets of "C1" bahiagrass and subsequent cell suspension culture. (A) Callus formation from leaflet on culture medium. (B) Yellowish calli formation. (C) and (D) Formations of embryogenic calli (white color) and nonembryogenic calli on the surfaces of the same callus. (E) Undifferentiated small pieces of cytoplasmically dense cells in typical suspension culture. (F) Brown color of large pieces in the same suspension culture. l, leaflet; n, nonembryogenic calli; e, embryogenic calli. Bars = 50 μm and 100 μm in (E) and (F), respectively.

that of previous reports on Paspalum notatum (Marousky and West, 1990; Chen *et al.*, 2001), *Panicum maximum* (Lu and Vasil, 1985; Akashi and Adachi, 1991) and *Dactylis glomerata* (Gray *et al.*, 1984). So, they were considered as embryogenic calli and subsequently were used as the starting materials of cell suspension culture. After 2 weeks of culture, the suspension cells could be classified into two kinds of calli, small, starch-containing embryogenic cells with dense cytoplasm (**Fig. 2E**) and large, vacuolated nonembryogenic cells. When the large cells were subcultured in the same medium, they became brown color (**Fig. 2F**) and the growth was decreased. In contrast, the small ones which were subcultured in the same medium, showed uniform size and white color with vigorous growth. The appearance of two kinds of calli was same as that of suspension cultures of *Paspalum dilatatum* (Akashi and Adachi, 1992).

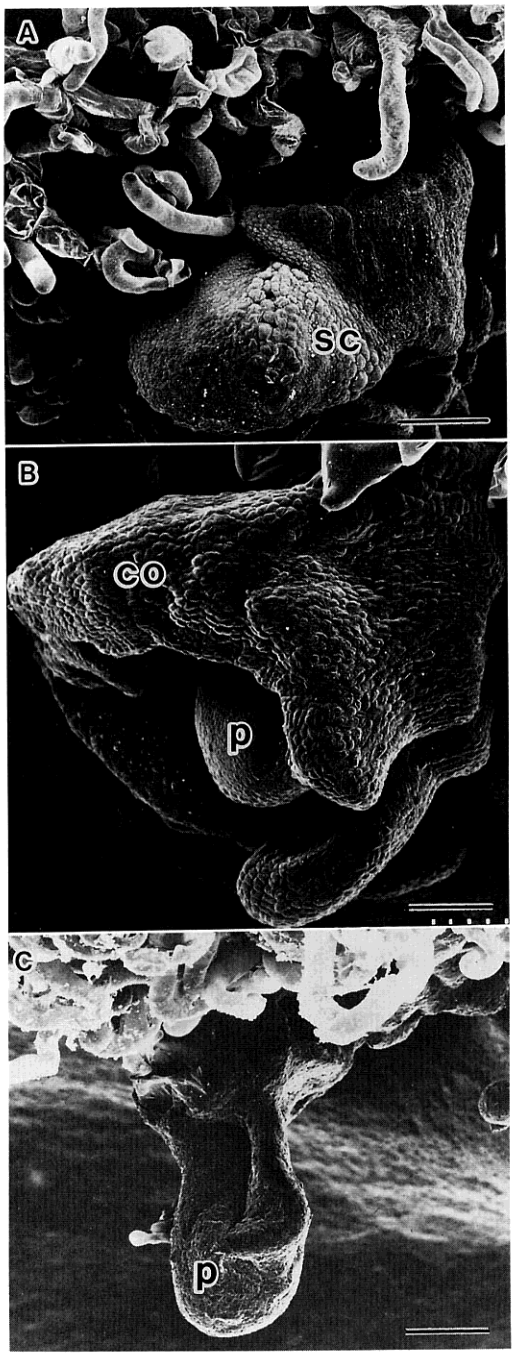

Fig. 3 Electron microscopy of somatic embryos. (A) Scutellum (SC); (B) Coleoptile (CO) and plumule appearance (P); (C) Elongation of plumule (P). Bars = 100 μm, 98 μm and 225 μm in (A), (B) and (C), respectively.

Plant regeneration from cell suspension culture

Preliminary experiments of plant regeneration from this callus culture indicated that the size of the calli placed on regeneration medium could affect shoot regeneration rates. As an attempt, we compared the number of shoots formed per callus piece using two different callus sizes (callus diameter of ≦ 2 mm or ≧ 4 mm). The shoots of total and per callus piece from the ≦ 2 mm callus were 5.9 times higher than that of the ≧ 4 mm callus (**Table 1**).

Table 1. Regeneration performance of "C1" bahiagrass suspension culture at 6 weeks after transfer to regeneration midium

Diameter of callus piece	No. of callus pieces started	Total No. of shoots formed	Average No. of shoots per callus piece
mm			($\chi \pm$ SE)
≦ 2	100	207	2.07 ± 0.15
≧ 4	100	35	0.35 ± 0.09

Significant difference of shoot regeneration between the two kinds of calli was observed. Combining the histological observations (**Fig. 2E, F**) and shoot regeneration rates (**Table 1**) of the calli of ≦ 2 mm and ≧ 4 mm, it is clear that compact and small size of calli (≦ 2 mm) is more optimal for plant regeneration than the large size and vacuolated calli (≧ 4 mm).

The calli of 2 mm in diameter were selected out from suspension cultures and placed onto solidified regeneration medium for plant regeneration (**Fig. 4A**). After two to three weeks of culture, the embryogenic calli formed anthocyanin pigment and the semitransparent plumules appeared (**Fig. 4B**). It followed a same course to that of direct regeneration from leaflets via somatic embryogenesis (Chen *et al.*, 2001). The plumules appeared from the embryogenic calli grew to shoots (**Fig. 4C**) after another 3 weeks of culture. When these shoots were removed from the calli and transferred onto photohormone-free MS medium, the roots and leaves were developed (**Fig. 4D**). After acclimation described in M & M, regenerated mature plants had typical morphology (**Fig. 4E**). There was a 100% survival rate among the transferred plants. As described above, a simple and efficient culture system was established within 60 days from suspension culture to plant regeneration.

In order to determine the ability of differentiation from cell suspension cultures after long-term subcultures, we used the same suspension culture subcultured at intervals of 1 week and measured the rate of regeneration after 2, 3, 6 and 12 months. The values of calli with greenish spots and shoots, and shoots per callus piece were almost same level, though those somewhat decreased gradually as the days increased (**Table 2**). So, we believe that the differentiation ability can be kept during 12 months by using this suspension culture system, at least. Now, the suspension cultures are being continued.

By the procedure of suspension culture system established in this study, complete plants via somat-

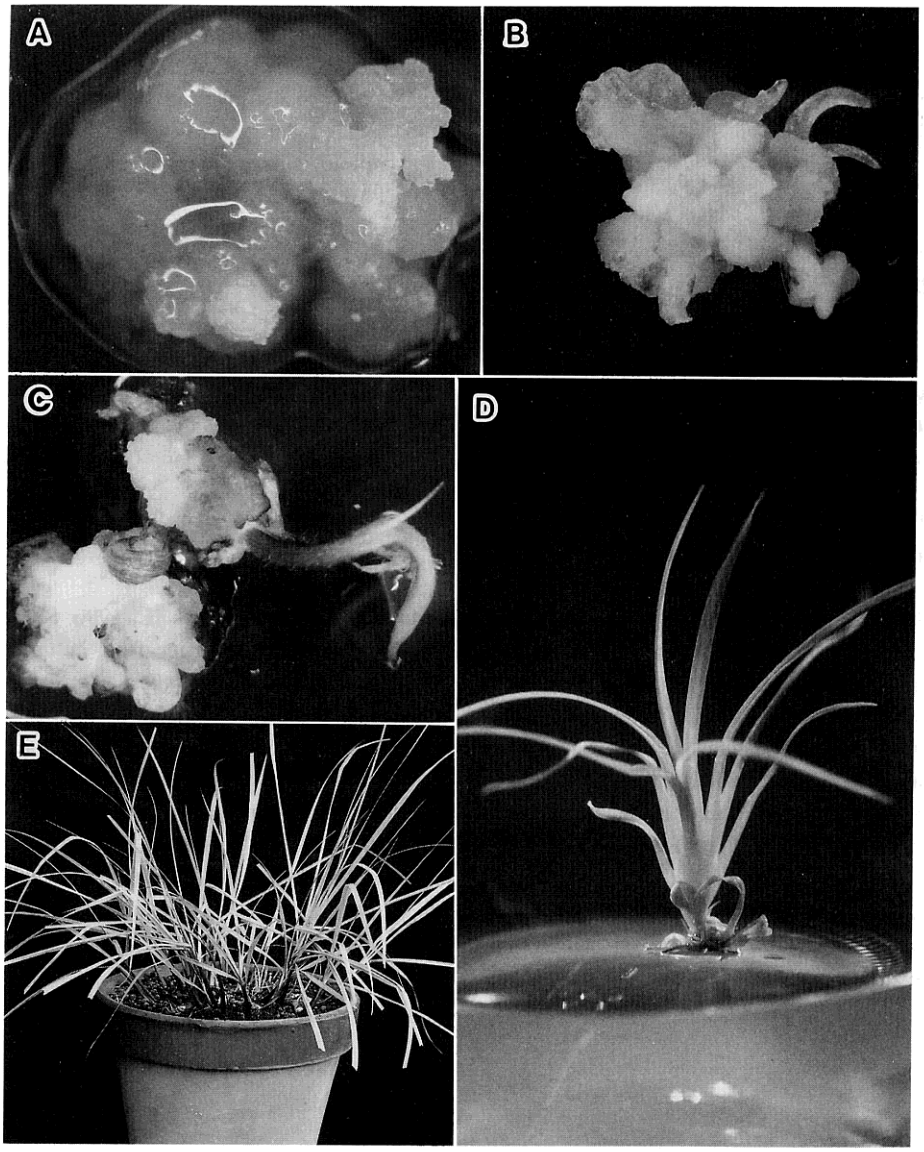

Fig. 4 Plant regeneration from suspension cell cultures derived from leaflets of "C1" bahiagrass. (A) Callus pieces of suspension culture were placed onto solidified regeneration medium; (B) Germinating embryos with expanding plumules; (C) Shoot development; (D) Plant regeneration after shoots were moved onto photohormone-free MS medium for rooting; (E) Potted mature bahiagrass plants.

Table 2. Subculture months affect differentiation of plants from embryogenic suspension culture derived from leaflets culture of "C1" bahiagrass

Subcultures	No. calli	% green spots	% shoots	No. shoots/ callus
months				($\chi \pm$ SE)
2	90	59	25.6	1.62 ± 0.25
4	90	61	24.9	1.74 ± 0.21
6	90	60	24.3	1.65 ± 0.23
12	90	58	23.5	1.56 ± 0.19

ic embryogenesis were obtained according to selection of embryogenic calli in the size of 2 mm in diameter, within 60 days after suspension culture. This system has some advantages of not only the leaflets used as materials, which allow the cultures all the year possible (Chen *et al.*, 2001), but also differentiation ability kept for as long as over 12 months by suspension culture. This system of constant and rapid regeneration from suspension culture derived from leaflets of sexual "C1" bahiagrass

facilitate to do cell and gene manipulations anytime, and will be extensively used in producing transgenic plants of monocots.

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