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Somatic Embryogenesis and Plant Regeneration from Leaflets of "Nanou" Bahiagrass

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Abstract

It is an important basic step in developing genetic modification techniques to identify relevant simple and efficient tissue culture and plant regeneration systems for agronomically important cultivars. Culture conditions for somatic embryogenesis and plant regeneration from leaflet tissues of "Nanou" bahiagrass (*Paspalum notatum* Flugge L.) are described. Cultures were done for callus formation on Murashige and Skoog (1962) medium (MS) containing 2,4-dichlorophenoxyacetic acid in the dark, and for plant regeneration on MS medium containing kinetin and giberellic acid in 16 h light. Two kinds of yellowish and white calli were obtained from the leaflets. In the continuous cultures, embryogenic calli and somatic embryogenesis were initiated from yellowish calli, but not from white calli. The plants were produced with multiple germinating embryos. We have therefore established a simple and efficient tissue culture and plant regeneration system available for genetic engineering.

Introduction

Recently, foreign genes have been introduced into monocots by particle bombardment of immature embryos or callus cultures (Fromm *et al.*, 1990; Vasil *et al.*, 1992; Zhong *et al.*, 1993). Though in monocot transformation some successes were made by introducing DNA into totipotent protoplasts from suspension cultures and regenerating plants from these protoplasts, the utilization of callus culture provides some advantages compared with protoplast experiments. For example, 1) callus culture is easier to maintain and less susceptible to contamination problems than suspension culture, 2) gene introduction experiments using particle bombardment procedure are simpler than techniques using protoplast preparations. Moreover, for the initial materials to produce callus and plants, the most important factor is that the material used can be obtained in any season, at any time, and be easy and efficient for regeneration. Some materials used to produce callus have been successful, e.g. mature seeds, immature seeds and/or embryos (Fromm *et al.*, 1990; Vasil *et al.*, 1992; Akashi *et al.*, 1992). However, all of these materials are reproductive organs, so they are limited to collection in certain seasons.

We are interested in establishing a system of somatic embryogenesis and plant regeneration from leaflets of warm-season forage grasses. Now, we are attempting to develop methods for the gene transformation in "Nanou", a cultivar raised from a population of 27 bahiagrass accessions introduced from the USA using a recurrent restrictive phenotypic selection scheme (Komatsu *et al.*, 1991). The objectives of this study were to establish a simple and efficient plant regeneration system from leaflet tissue of "Nanou" bahiagrass, which can be used as material in any season and at any time.

Materials and Methods

Plant materials

Potted, greenhouse grown mature "Nanou" bahiagrass plants (*Paspalum notatum* Flugge L.) were used as culture materials for explants. Leaflet tissues were taken from terminal tillers arising at the end of stolons. Two to 3 cm of the leaflets were isolated by trimming the leaves and excising the parts of the leaf-stem tissue 1 cm above the root base which contains the basal meristem and basal leaf tissue. The outer four to five leaves were removed. The remaining tissues were cut into 5-

mm lengths and washed in streaming tap water for 20-30 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 time. The explants were then placed on Murashige and Skoog (1962) medium (MS) for callus formation with one of the cut surfaces in contact with the medium.

Culture conditions

To determine the effects of hormones on callus formation, the MS medium containing 2, 5, 10, 20 mg/*l* 2,4-dichloro-phenoxyacetic acid (2,4-D), 30 g/*l* sucrose, 2.5 g/*l* Gellan Gum (Wako Pure Chemical Industries, Ltd. Japan) was used and the cultures were incubated in dark at 25 ℃.

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 yellowish - calli - derived calli and their rates were observed on the 9th week. The selected embryogenic calli were then transferred onto 5 kinds of MS media complemented with kinetin (Kin) and giberellic acid (GA3) for plant regeneration at 25 ℃ in light condition (photon flux density = 33.3 μ mol m-2s-1, 16 h). The rates of green spot and shoot regeneration were observed at the 12th week.

Plant acclimation

The shoots were removed from the cultures and rooted on hormone-free MS medium. The rooted plants were potted in washed sand and covered with sealed plastic vinyl bags to keep full humidity at 25 ℃ in light condition (photon flux density = 33.3 μ mol m-2s-1, 16 h). As the plants grew vigorous, the bags were poked with chopsticks to allow air enter inside the bags until to the plants self-supported.

Microscopic studies

Embryogenic calli were prepared for scanning electron microscopy by fixing in FAA solution (ethanol 45%, acetic acid 2.5% and formalin 2.5%) for 24 h. Sample fixation was followed by tissue dehydration by an ethanol series (50% 60%, 70%, 80%, 90%, 99.5% and 100%) and twice in 100% 3-methylbutyl acetate. Thereafter, the samples were critical point dried, and sputtered with gold prior to scanning electron microscopy (HITACHI S4100).

Results and Discussion

Callus formation and embryogenic calli

After sterilization the leaflet explants were usually brown in color (**Fig. 1a**). They were placed on the medium and grew with watery, yellowish color from three days (**Fig. 1b**). It seemed as if the explant was covered by yellow liquid paste. The calli emerged from two sides of the center of the explant (**Fig. 1c, d**). The colors of calli were different between the two sides of the tissue. The side in contact with the medium was brown and the opposite side was yellowish. At three weeks of cultures, both calli were of white or yellowish color and the outside of the explants became brown. At this point, the rate of callus formation on the medium with 5-mg/*l* 2,4-D was 25.8%, highest among the four combinations of hormones (**Table 1**). After another three weeks of culture, the yellowish callus covered with weak yellow-white color (**Fig. 2a**) lost its color, and became nodular and translucent, white calli were formed on the surface (**Fig. 2b**). When the callus with yellowish

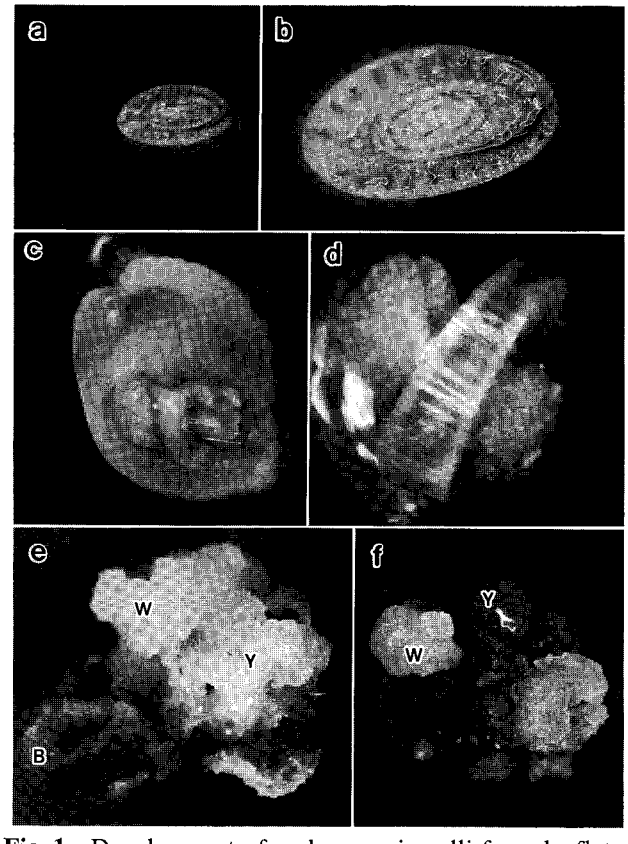

Fig. 1 Development of embryogenic calli from leaflets. (a) Placed 5 mm length leaflet on callus medium. (b, c, d) Calli formation on leaflets three days, seven days and two weeks, respectively, after placing. (e, f) Both of calli formation, white and yellowish-white, and brown outside leaflets. W, white color calli; Y, yellowish-white color calli. B, leaflet with brown color.

Table 1. The effects of hormones on callus formation from leaflets of "Nanou" bahiagrass*

2,4 − D (mg/*l*)	No. explants cultured	No. explants forming callus (%)	No. of explants forming embryogenic callus (%)
2	199	46 (23.1)	23 (28.3)
5	178	46 (25.8)	8 (17.4)
10	174	24 (13.8)	2 (8.3)
20	120	36 (3.0)	0 (0)

*1) MS medium was used as basic medium; 2) Callus formation and embryogenic calli were investigated at 3 and 9 weeks after the start of culture, respectively.

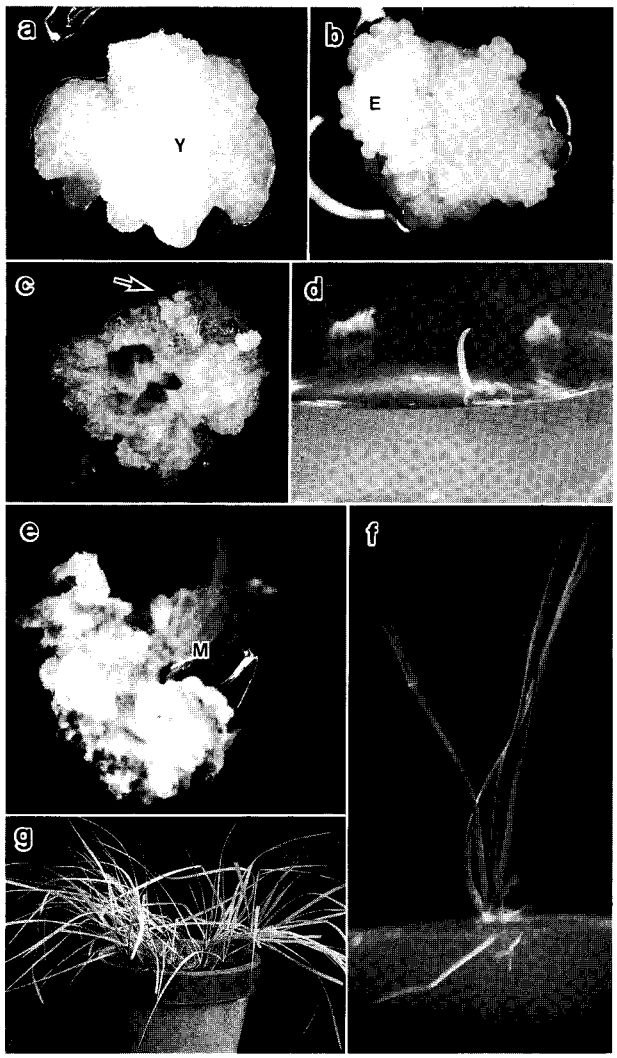

Fig. 2 Somatic embryogenesis and plant regeneration. (a) Calli with yellowish-white color for subculture. (b) Formation of nodular and white color embryogenic calli on the surface of the yellowish-white color calli after another 3 weeks of culture. (c) Green spot (arrow) and anthocyanin accumulated in embryogenic calli on regeneration medium. (d) Plumules regenerated on embryogenic calli. (e) Multiple shoot regeneration. (f) Complete plant regeneration on hormone-free MS medium. (g) Successful plant acclimation. Y, yellowish-white color calli. E, embryogenic calli. M, Multiple shoot regeneration.

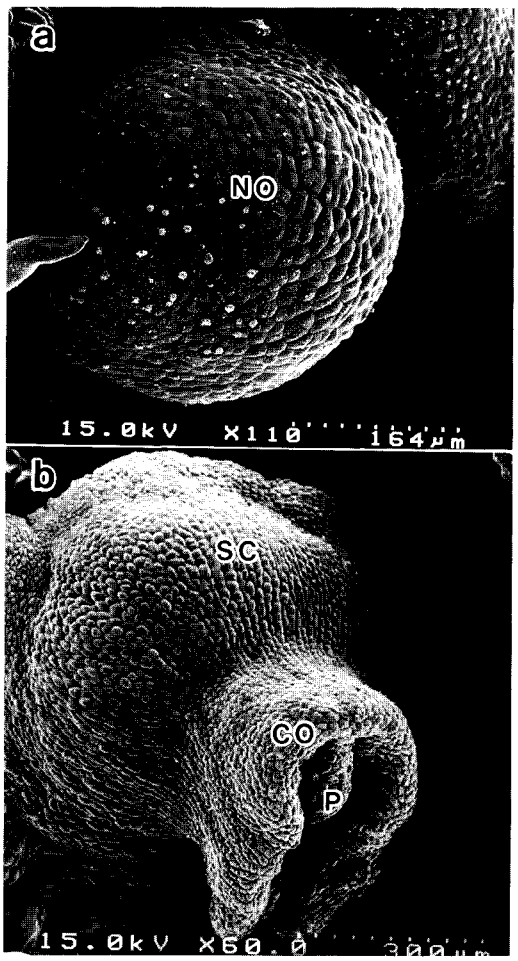

Fig. 3 Scanning electron microscopy of somatic embryo. (a) Nodular embryogenic tissue (NO). (b) Scutellum (SC), coleoptile (CO) and germination of plumule (P).

color was observed, it seemed as being contaminated. However, as the cultures were continued, the yellowish color was lost. This may be a bahiagrass-specific characteristic.

In order to improve somatic embryogenesis, the calli of **Fig. 2b** were observed with a scanning electron microscopy. Embryogenic structures with nodes (**Fig. 3a**), and somatic embryo with scutellum, coleoptile and germinated plumule (**Fig. 3b**) were observed. These structures were similar to those previously reported in *Panicum maximum*

Table 2. The effects of hormones on plant regeneration from embryogenic calli of "Nanou" bahiagrass*

Kin (mg/*l*)	GA3 (mg/*l*)	No. embryogenic calli	No. calli with GS (%)	No. calli with shoots (%)	Total no. plants regenerated	No. shoots per callus
0	0	136	80(58.8)	38(47.5)	71	1.87
0.1	0.1	112	46(41.1)	20(43.5)	34	1.7
0.1	1	96	55(57.3)	27(49.1)	51	1.89
1	0.1	108	76(70.4)	21(27.6)	27	1.29
1	1	108	96(88.9)	18(18.8)	39	2.17

*1) MS medium was used as basic medium; 2) GS (green spots) and shoots were investigated at 3 and 6 weeks after embryogenic calli were transferred to regeneration medium, respectively.

Jacq. (Lu and Vasil, 1985), *Paspalum notatum* Flugge (Marousky and West, 1990) and *Paspalum dilatatum* Poir (Akashi and Adachi, 1992). In preliminary experiments, when the nodular and white color calli were moved onto regeneration medium for culture, somatic embryos were initiated. So the calli were named as embryogenic calli. The embryogenic callus showed white, scalloped tissue, which were similar in structure to that described for orchardgrass (Gray *et al.*, 1984) and guineagrass (Akashi and Adachi, 1991; Shatters *et al.*, 1994). The rate of embryogenic calli was 28.3% (**Table 1**) and they were initiated on the calli obtained from MS medium with 2 mg/*l* 2,4-D. The low percentage of embryogenic calli formation from leaflets may represent the small subpopulation of the "Nanou" plants genetically predisposed to form somatic embryos. On the other hand, the white color calli formed in the first three weeks, were also continued in culture but they did not become embryogenic calli, and could not initiate somatic embryos in the end. So, they were labeled as nonembryogenic ones (ca. 70%) in this study.

Plant regeneration and somatic embryogenesis

From embryogenic calli initiated from leaflets, small size and white color calli were selected and propagated in the same medium by subculture every two weeks. The propagated calli were divided into 20 mg each and moved onto regeneration medium for shoot regeneration. After two to three weeks of culture, the embryogenic calli formed anthocyanin pigment and green spots emerged from them (**Fig. 2c**). The plumules from the embryogenic structures (**Fig. 2d**) grew to multiple shoots (**Fig. 2e**) after another 3 weeks of culture. When these shoots were removed from the calli and transferred onto hormone-free MS medium, the leaves and roots developed (**Fig. 2f**). After acclimation described in M & M, regenerated mature plants had typical morphology (**Fig. 2g**).

Among the five media for plant regeneration, although they all gave higher green spot regeneration rates from 41.1% to 88.9%, 1 mg/*l* Kin and 1 mg/*l* GA3 medium gave the highest rates (88.9%) (**Table 2**). From the green spots, however, shoot regeneration rates were from 18.8% to 49.1% lower than that of the spots. The medium with 0.1 mg/*l* Kin and 1 mg/*l* GA3 gave the highest rates (49.1%) of shoot regeneration among the five media. Here, it should be noted that hormone-free medium gave 47.5%, a higher shoot regeneration rate. Especially, 1mg/*l* Kin and 1 mg/*l* GA3 medium that gave the highest green spot regeneration rates, in contrast, gave the lowest rates of shoot regeneration. **Table 2** shows the trend that the rates of shoot regeneration were contrary to that of green spots, except the 0.1 mg/*l* Kin and 0.1 mg/*l* GA3 medium. This result may indicate that the process from green spot to shoot regeneration does not need hormones, like Kin, which stimulate cell division. As before the process, differentiation almost was finished, just cell elongation may be needed from spot to shoot regeneration. The number of shoots per callus were from 1.29 to 2.17 among the five media. The shoots grew into complete plants at a rate of 100% with a 100% survival rate in greenhouse for the transferred plants.

Selection for embryonic and regenerable calli

High embryogenic and regenerable calli were selected from heterogeneous calli by morphology comparison. In our experiments, both of yellowish and white calli were obtained directly from leaflets. White calli could not differentiate into embryogenic calli and then further into shoots. However, when the yellowish calli were cultured continuously, the yellowish color was gradually lost. On the surface of the calli, nodular, white and hard embryogenic calli were formed. The characteristics of nonembryogenic calli, yellowish-white and opaque obtained in this study, were same as that of bahiagrass callus

cultures of Shatters *et al.* (1994). However, the differences between our experiments and theirs were that, after the yellowish callus were further cultured, they became embryogenic calli (**Fig. 2a, b**). 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).

Using the plant regeneration system established in this study, complete plants were obtained in 4 months via somatic embryogenesis based on a selective system of embryogenic calli from leaflets. Another advantage of this study was that leaflets were used as materials for plant regeneration which allows cultures all the year round as leaflets can be collected at any season unlike some reproductive tissues such as immature inflorescence, immature and/or mature embryo or seeds. This system of rapidly regenerating plants from "Nanou" bahiagrass leaflet culture and selective subcultures of embryogenic calli can be the utilizated in genetic engineering experiments, especially for developing transgenic plants. Recently, attempts on apomixis-specific gene (Chen *et al.*, 1999a,b) transformation into the selected yellowish calli are in progress.

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