Embryogenic Callus Formation from Protoplasts Derived from Suspension Cells of Apomictic Guineagrass (*Panicum maximum* Jacq.)

Ryo Akashi, Siegfried Lachmann¹, Franz Hoffmann² and Taiji Adachi

*Faculty of Agriculture, Miyazaki University, Miyazaki, Japan 889-21*
¹ Taihi Eurepe B.v., Hoofdweg 19, 1424 FC De Kwakel, The Netherlands
² Department of Developmental and Cell Biology, University of California, Irvine, California 92717, USA

Summary

Protoplasts were isolated from suspension cells of apomictic guineagrass (*Panicum maximum* Jacq.). The suspension culture used as donor material was originally initiated from immature embryo-derived embryogenic callus. Prior to protoplast isolation, suspension cells were conditioned with Murashige and Skoog (MS) liquid medium without sucrose and growth regulators. This pretreatment lead to a dramatic increase in protoplast yield and colony formation. Cell division and colony formation from such pretreated protoplasts were found to be best in agarose-solidified modified KM89 medium. Protoplast derived colonies developed in callus on solidified MS medium supplemented with 1 mg/l 2,4-D. After 2 months in culture, calli formed compact white nodular structures some of which developed into somatic embryos. Although some of the somatic embryos developed small leafy structures, whole plants could not be regenerated.

Key words: apomixis, embryogenic callus, guineagrass, *Panicum maximum* Jacq., protoplast culture, suspension cells.

Introduction

Guineagrass (*Panicum maximum* Jacq.) is one of the most important warm-season grasses for forage production in tropical and subtropical areas in the world. Nutritional value and dry matter yield of guineagrass are higher than those of other warm-season forage grasses. Recently it became known that guineagrass is effective in suppressing the density of nematodes (*Meloidogyne* spp.) in the soil when utilized as green manure crop (Shimizu and Sato 1986). However, the cultivation of this grass is limited because of unstable seed germination, slow initial growth, reduced seed yield due to shattering and still insufficient nutritional value (Nakajima and Mochizuki 1983). Therefore, genetic improvement of the species is mandatory. The normal reproduction mode of guineagrass is by facultative apomixis (Warmke 1954). Improvement through conventional breeding has been extremely difficult because of the apomictic barrier to hybridization, or the problem of controlling variation in the progeny of facultative apomicts. Thus, genetic improvement of this grass can be more easily expected from *in vitro*-techniques, especially, through the introduction of tissue culture-derived variation.

*In vitro* technology, such as the regeneration of fertile plants from cultured cells or tissues is still problematic in grass species. In guineagrass, plants have been regenerated from somatic embryos originated from leaf bases, immature embryos, and immature inflorescences (Lu and Vasili 1981 a, 1982). Akashi and Adachi (1991) regenerated numerous plants from immature embryo-derived callus by selecting a highly embryogenic cultivar and demonstrated a possible positive correlation between “regeneration” and degree of apomixis. Plant regeneration has also been achieved from suspension cells (Lu and Vasili 1981 b). The same authors obtained embryos from cell suspension-derived protoplasts more than a decade ago (Lu and Vasili 1981) but regenerated plantlets died after formation of the first three leaves and further progress has not been reported since then. The objective of this study was to investigate if the regeneration problem reported in guineagrass protoplasts can be overcome by using a preselected highly embryogenic cultivar which is also of highly apomictic nature.

Materials and Methods

1. Establishment of suspension cultures

Embryogenic calli were induced from immature embryos of the highly apomictic cultivar ‘Petrie’ of guineagrass (*National Institute of Agrobiological Resources*, Miyazaki, Japan) on MS (Murashige and Skoog 1962) medium with 10.0 mg/l 2,4-D and 10 % coconut water solidified with 0.2 % Gellan Gum (Wako Pure Chemical Industries, LTD., Osaka, Japan) as described previously (Akashi and Adachi 1991). After 2 or 3 subcultures, cell suspensions were started from embryogenic calli in MS liquid medium with 1.0 mg/l 2,4-D. Cultures were incubated on a rotary shaker (80-90 rpm) at 25-27°C in the dark and subcultured at intervals of 7 days. Suspension cultures became fully established 8-10 weeks after initiation.

2. Protoplast isolation and culture

Approximately 1 g of suspension cells, collected 3 days after transfer to fresh medium, was pretreated in 10 ml of MS medium without sucrose and growth regulators at

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80-90 rpm in the dark for 12 h before protoplast isolation. For the first isolation, the suspensions were at least 6 months old and are successfully used until today at about 3 years. Following the pretreatment, cells were incubated in 10 ml of enzyme solution containing 2.0 % (w/v) Cellulase ‘Onozuka’ RS (Yakult Pharmaceutical Co., Tokyo, Japan), 0.1 % (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo, Japan) in PFM (protoplast washing medium (Akashi and Adachi 1992), 0.1 % CaCl₂, 0.5 M Mannitol, pH 5.7-5.8). The mixture was kept on a shaker for 3-4 h at 25-27 °C in the dark. After enzyme incubation, the suspension was passed through 59 and 33 μm nylon mesh. Protoplasts were washed three times by centrifugation in PWM at 100g for 5 min and finally suspended in protoplast medium (see below). The viability of protoplasts was assessed using fluorescein diacetate (Widholm 1972). Protoplasts were cultured in the dark at 25-27 °C by using the agarose-bead method either with or without nurse cells in 6 cm petri dishes (Kozuka et al. 1987). A modified KM8p medium (Kao and Michayluk 1975) was used as the initial culture medium. This modified version contained no free amino acids, nucleic acid bases, riboflavin, vitamins A and B₁₂, casein hydrolysate and coconut water, but 0.4 M glucose, 1250 mg/l sucrose and NAA, 2,4-D, or zeatin at various concentrations (Akashi and Adachi 1992). The protoplast suspension in modified KM8p protoplast medium was mixed with an equal volume of 2.0 % (w/v) molten agarose (Sea Plaque LGT; FMC Co., Rockland, Me., USA) as described in Akashi and Adachi (1992). For nurse cultures, agarose plates were cut into 4 and 6 blocks and transferred individually into 6 cm dishes containing 4 ml of the liquid protoplast medium. Approximately 200 mg of suspension cells of the parental line were added as nurse cells. Agarose blocks were transferred to fresh medium after 10 days with nurse cells completely removed by washing with culture medium. Plating efficiency was estimated after 20 days as the percentage of protoplasts that had formed cell colonies which were visible microscopically. After 4 weeks of culture colonies were cut out of the agarose block and transferred to MS medium containing 1.0 mg/l 2,4-D and 0.1 % Gellan Gum. About 1 month later, the protoplast-derived calli were placed on MS hormone-free medium or on MS medium supplemented with 0.2 mg/l 2,4-D (Lu and Vasil 1981 c). Callus-derived somatic embryos were transferred to MS medium with 1.0 mg/l each of kinetin and GA₃ (Akashi and Adachi 1991). All colonies and calli were cultured under continuous fluorescent light (approx. 3,000 lux) at 25-27 °C. All media were adjusted with NaOH or HCl to pH 5.8 and autoclaved at 121 °C for 15 min with the exception of KM8p medium which was filter-sterilized.

Results and Discussion

Cell suspensions of guineagrass were successfully established in MS medium containing 2,4-D. Cell clusters were initially relatively large and compact. However, after three months clusters were smaller and starch-containing with dense cytoplasm, a phenotype typical for embryogenic cells (Fig. 1 a).

Initial experiments on the isolation of protoplasts from suspension cells with enzyme solution in 0.3 M osmoticum as previously reported by Lu et al. (1981) resulted in yields of only 6 x 10⁶ to 7.5 x 10⁵ protoplasts/g FW, which is too low for substantial further experimentation. Therefore, experimental conditions were sought that would produce at least 10⁶ viable protoplasts/g FW. Previous to protoplast isolation, suspension cells were treated with MS liquid medium without sucrose and growth regulators, and subsequently, the cells were suspended in enzyme solutions of different osmotic potentials. The osmotic value of the enzyme and washing solutions was created with mannitol, and, although this adjustment was extremely important, a broad concentration range (0.4-0.6 M) was suitable for stabilizing the protoplasts (Fig. 2). As a result, the yield of protoplasts was between 4.0 x 10⁶ and 5.0 x 10⁶ protoplasts per g suspension cells. Furthermore, more than 90 % of such pretreated protoplasts were viable, as determined by staining with fluorescein diacetate (date not shown).

The average diameter of protoplasts was 20-30 μm (Fig. 1 b). The first cell divisions occurred after 5-7 days, and microscopical colonies were observed after 10-15 days of culture (Fig. 1 c-g). These colonies grew vigorously and developed calli about 0.5 mm in diameter within 20 days (Fig. 1 h).

Table 1 shows the effect of nurse culture on colony formation of P. maximum protoplasts. In previous reports on other Gramineae nurse cells were absolutely necessary to induce division and to obtain reproducible colony formation (Kozuka et al. 1987, Akashi and Adachi 1992). In guineagrass, however, colony formation occurred more frequently in the absence of nurse cells. Individual colonies grew to 1.0 mm in diameter within 40 days of culture. Colony formation was obtained in 5 of the 6 culture media examined, but the protoplast plating efficiency varied (Fig. 3). The highest plating efficiency was obtained in protoplasts cultured in PC 5 medium (0.89 %), the second highest in PC 6 (0.56 %). Both media are KM 8 p-based containing 1.0 mg/l 2,4-D or 1.0 mg/l NAA, 0.2 mg/l 2,4-D and 0.5 mg/l zeatin, respectively. All MS-based media investigated turned out to be much less successful. No quantitative data are given in the report by Lu et al. (1981). According to these authors vigorous cell division occurred in a KM 8 p-based medium at 0.1-1.0 mg/l 2,4-D, and dense and compact embryogenic aggregates formed at 0.25 and 0.5 mg/l 2,4-D.

The colonies, after transfer to soft MS medium containing 1.0 mg/l 2,4-D and 0.1 % Gellan Gum, continued to proliferate and after about 1 month formed embryogenic tissues with white nodule and compact structures (Fig. 1 h). For germination of somatic
Table 1. Negative effect of nurse cells on colony formation in guineagrass protoplasts

<table>
<thead>
<tr>
<th>Cell colonies formed per $5 \times 10^5$ cultured protoplasts$^1$</th>
<th>Total</th>
<th>Colony formation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.793</td>
<td>0.56</td>
</tr>
<tr>
<td>Nurse culture$^2$</td>
<td>928</td>
<td>0.19</td>
</tr>
</tbody>
</table>

$^1$ Obtained after 20 days in culture, mean of 3 independent experiments with 3 or 4 replications each, PC 6 medium.
$^2$ The mixed nurse method was used (Koyzuka et al. 1987).

Embryogenic callus formation from suspension culture-derived protoplasts of guineagrass

Fig. 1. Formation of embryogenic callus from protoplasts derived from suspension cells of guineagrass (P. maximum).
(a) Aggregates of cytoplasmically dense, starch containing cells. (b) Freshly isolated protoplasts from suspension cells after pre-treatment in PTK solution. (c-f) Cell division and colony formation from protoplasts after 3, 4, 7 and 14 days of culture, respectively. (g) Numerous visible colonies formed from protoplasts in the agarose plating method after 20 days of culture. (h) Somatic embryo formation on protoplast-derived callus. Bars: 50 $\mu$m in (a-b); 30 $\mu$m in (c-f); 1 mm in (h).

embryos, small clumps of polyembryonic callus were placed on MS hormone-free medium, on MS medium supplemented with 0.2 mg/l 2,4-D (Lu et al. 1981) or on MS medium with 1.0 mg/l each of kinetin and GA$_3$ (Akashi and Adachi 1991). Two weeks after transfer, the explants had grown slowly on all media with browning of the callus portion of the tissue. Immediate transfer to fresh medium and subsequent subcultures at short intervals were carried out in an attempt to rescue the explants. However, only occasionally leafy structures developed but browning of the material continued and finally led to the death of the cultures.

Our experiments did not overcome the regeneration barrier reported by Lu et al. (1981) since protoplast-derived regenerates turned out to be arrested at a similar stage as reported previously. By selecting a highly embryogenic and apomictic cultivar and by using a more advanced protoplast culture technique we were able to
improve plating efficiency and embryo formation rate (as judged by the figures given in Lu et al. 1981) but did not produce plants. In our study, protoplast isolated from 6 months old suspensions were initially cultured, and results did not change during two years of continuous protoplast isolation. Lu et al. (1981) used 18 months old cultures as protoplast source. If the age of the cell suspension is causing the regeneration problem we have little time to play with since it takes about 3 months to establish a fast growing embryogenic culture and at least another 2 months to optimize protoplast isolation and culture conditions. This leads to the conclusion that for some grass species the overall successful approach of isolating and culturing protoplasts derived from embryogenic cell suspensions might not be a solution. Of course, two negative reports, although 13 years apart, are not conclusive, but the possibility exists that the arrested embryos/plantlets are of immanent cause. We are now concentrating on developing cell suspensions from mature embryos. If such a system would regenerate plants, as it does in rice (Fujimura et al. 1985) it would have the additional advantage of unlimited availability of mature seeds. Immature zygotic embryos, as used in this study, are restricted to a very short season.

**Literature Cited**


