PROTOPLAST PRODUCTION FROM NAPIER GRASS AND PEARL MILLET TRIPLOID HYBRIDS

Obtenção de protoplastos de híbridos triplóides entre o capim-elefante e milheto

Ana Luiza de Oliveira Timbó[1], Lisete Chamma Davide[2], José Eduardo Brasil Pereira Pinto[3], Antônio Vander Pereira[4]

ABSTRACT

The objective of this work was to obtain protoplasts from napier grass and pearl millet triploid hybrids as a basis for future studies on chromosomal duplication. Explants were taken from mesophyll of *in vitro*- and *in vivo*-cultured plants or from calli of two triploid hybrids (H1 and H2), which were treated with enzymatic solutions containing different concentrations of cellulase R-10 (0.5, 1.0, 1.5 and 2.0%) with an additional 0.2% macerozyme and 0.1% driselase or 1.0% pectolyase Y-23 and 0.5% hemicellulase. Enzymatic digestion was monitored once every hour for five hours. Protoplasts were obtained from *in vitro* and *in vivo* leaflets of both triploid hybrids, and *in vitro* leaflets were the best explant sources. The quantity of produced protoplasts varied according to the hybrid, the enzymatic solution and the treatment time.

Index terms: *Pennisetum purpureum*, *Pennisetum glaucum*, *In vitro* cultivation, Interspecific hybrids, Forage.

RESUMO

Objetivou-se, neste trabalho, a obtenção de protoplastos de híbridos triplóides entre o capim-elefante e o milheto como base para futuros trabalhos de duplicação cromossômica. Foram utilizados explantes de mesofilo de plantas cultivadas *in vitro* e *in vivo*, ou de calos de dois híbridos triplóides (H1 e H2), os quais foram tratados com soluções enzimáticas em diferentes concentrações da enzima celulase R-10 (0,5; 1,0; 1,5 e 2,0%), acrescidas de 0,2% macerozyme e 0,1% driselase ou 1,0% pectolyase Y-23 e 0,5% hemicelulase. A digestão enzimática foi monitorada a cada hora durante 5 horas. Obtiveram-se protoplastos a partir de folhas *in vitro* e *in vivo* dos dois híbridos triplóides, sendo as folhas *in vitro* as melhores fontes de explante. A quantidade de protoplastos variou em função do híbrido, da solução enzimática e do tempo de tratamento.

Termos para indexação: *Pennisetum purpureum*, *Pennisetum glaucum*, cultivo *in vitro*, híbridos interespecíficos, forrageiras.

(Received in september 11, 2008 and approved in may 14, 2009)

INTRODUCTION

Napier grass is largely used to feed the livestock, especially cattle, sheep and goats. It also has great energetic potential for charcoal, bio-oil and biogas production, as well as for electricity in thermoelectric power plants and rural properties. In addition, short and purple cultivars can be employed in landscaping (Pereira et al., 2001; Mesa-Perez et al., 2005; Strezov et al., 2008).

In Brazil, napier grass is mainly used as forage, and although it is grown all over the country, there is a demand for improved cultivars that could better adapt to different edaphic-climatic conditions and utilization systems, due to the few existent plant breeding programs (Pereira et al., 2001).

Most of napier grass characteristics of forage importance can be improved by exploiting the variability within the species. However, based on the capability of this grass to exchange alleles with other *Pennisetum* Rich. species, the plant breeding program can use germplasm from species belonging to close genic groups like pearl millet (Pereira et al., 2001). This type of genetic combination tries to gather in the hybrid some of the desirable characteristics of pearl millet such as vigor, drought resistance, disease tolerance, forage quality and seeds size, whereas rusticity, aggressiveness, perennity, palatability and high dry matter yield are provided by napier grass (Schank et al., 1996; Jauhar & Hanna, 1998; Souza Sobrinho et al., 2008).

However, these two species have different ploidy levels: napier grass is allotetraploid (2n = 4x = 28, genomes A'A'BB), whereas pearl millet is diploid (2n = 2x = 14, genome AA). Hybridization between them produces an infertile triploid hybrid (2n = 3x = 21 chromosomes, genomes AA'B) and such infertility is the main barrier in

[1]Universidade Federal de Lavras/UFLA – Post office box 37 – Lavras, MG – 37200-000 – Scholarship holder of the Capes – oliveiratal@ yahoo.com.br
[2]Universidade Federal de Lavras/UFLA – Department of the Biology – Lavras, MG
[3]Universidade Federal de Lavras/UFLA – Department of the Agriculture – Lavras, MG
[4]Embrapa Gado de Leite – Juiz de Fora, MG

breeding programs, hindering its utilization in crossings and its propagation through seeds.

Fertility can be restored by chromosomal duplication using antimitotics (Hanna, 1981; Hanna et al., 1984; Abreu et al., 2006; Barbosa et al., 2007). Barbosa et al. (2007) induced chromosomal duplication in buds and shootings. However, when multicellular vegetative material was used *in vitro*, this duplication process resulted in the production of mostly mixoploid and a few stable hexaploid plants.

The utilization of protoplasts either for treatment with antimitotics (Zeng et al., 2006) or for fusion could help overcome the mixoploidy problem, producing stable hexaploid plants. Another advantage is that hybrids can be mass-produced after the protocol is optimized.

Thus, the aim of the current work was to develop a methodology for protoplast production from napier grass and pearl millet triploid hybrids as a basis for future studies on hybrid chromosomal duplication through both protoplasts fusion for allohexaploids formation and antimitotic treatment for autohexaploids formation.

MATERIAL AND METHODS

Treatments consisted of two triploid hybrids from napier grass (*Pennisetum purpureum* Schumach.) and pearl millet (*Pennisetum glaucum* (L.) R. Br.), designated H1 (CNPGL 91-2-5x M42) and H2 (Merker Pinda x M42); three vegetative materials (leaves from seedlings cultured *in vitro* and *in vivo*, and calli); eight enzymatic solutions [cellulase R-10 (0.5, 1.0, 1.5 and 2.0%) added of macerozyme (0.2%) and driselase (0.1%) or pectolyase Y-23 (1.0%) and hemicellulase (0.5%)]; and different incubation times (1, 2, 3, 4, and 5 hours).

The plant materials were taken from seedlings germinated in Plantmax® substrate in a greenhouse or germinated *in vitro*. For *in vitro* germination, seeds were disinfected with sulfuric acid 50% for 15 minutes and treated with alcohol solution 70% (v/v) for 1 minute. Then, seeds were immersed in sodium hypochlorite solution 50% (v/v) containing 2.5% active chlorine content during 15 minutes and washed three times with sterile distilled water in a laminar air-flow cabinet. Each seed was inoculated into a test tube containing 10 mL MS culture medium (Murashige & Skoog, 1962) supplemented with 3% sucrose. Cultures were kept in a growth chamber at 26 ± 2º C with 16 h light/8 h darkness and a total irradiance of 25 μmol m^2 s^{-1} provided by cool-white fluorescent lamps. Assays for protoplast production were carried out after 30 days of *in vivo* or *in vitro* cultivation.

Callus culture was obtained from three-cm basal explants taken from *in vitro* cultured seedlings of triploid hybrids and transferred to containers (250 mL) with 30 mL of MS medium plus 2.0 mg L^{-1} of the plant growth regulator 2,4-D (dichlorophenoxyacetic acid). Cultures were maintained in a growth chamber at 26 ± 2º C with 16 h light/8 h darkness and a total irradiance of 25 μmol m^2 s^{-1} provided by cool-white fluorescent lamps. For culture maintenance, calli were subcultivated in a new medium every 20 days, according to Passos & Kattermain (1994).

Enzymatic solutions were prepared through dilution in CPW (*cell protoplast wash*) medium which contained KH_2PO_4 (27.2 mg L^{-1}), KNO_3 (101 mg L^{-1}), $CaCl_2.2H_2O$ (1480 mg L^{-1}), $MgSO_4.7H_2O$ (246 mg L^{-1}), KI (0.16 mg L^{-1}), and $CuSO_4.5H_2O$ (0.025 mg L^{-1}), according to Frearson et al. (1973), supplemented with mannitol 13%. After pH adjustment to 5.6 with 5 mM 2-[N-morpholino]-ethanesulphonic acid (MES), solutions were filter-sterilized in Millipore filter membranes of 0.22 μm porosity.

The grass was planted in potting soil and kept inside the greenhouse. The primary explants were washed in running tap water for 30 min. The foliar segments were disinfected in 70% (v/v) ethanol for 1 min, immersed in 2.5% sodium hypochlorite solution for 10 min, and washed three times with sterile distilled water.

The protoplasts were obtained from 500 mg of *in vivo* and *in vitro* leaflets. Approximately 1-cm-long foliar segments were sliced into 1 mm strips. The plant material was then pre-plasmolized for 1 hour, in the absence of light, in 20 mL CPW + mannitol 13%.

Later, CPW + mannitol 13% solution was discarded using Pasteur pipettes and 20 mL of the enzymatic mixture was added. Each sample received different enzymatic treatments.

Protoplasts were obtained from calli by transferring 1 g plant material to a 10 mL CPW + mannitol 13% solution, for 1 hour, in the dark, to allow pre-plasmolisis. At the end of this period, 10 mL concentrated enzymatic solution was added, resulting in a final volume of 20 mL.

Enzymatic digestion occurred due to the 5-hour incubation of leaf and callus explants in the dark, at 40 rpm agitation and 26±2° C temperature. It was monitored once every hour, when a little aliquot was taken from each plate/treatment for protoplasts quantification under optical microscope in a Neubauer chamber. Evans blue stain at 0.05% was used to count viable protoplasts, i.e. those that excluded the stain.

RESULTS AND DISCUSSION

Protoplast yield was dependent on the employed plant material. Leaflets from plants cultured both *in vivo* and *in vitro* showed to be the best sources (Table 1 and Figure 1).

Table 1 – Quantification of viable protoplasts (x 10^4) produced from leaflets of seedlings cultured *in vitro* and *in vivo* and from calli of H1 and H2 triploid hybrids in different enzymatic solutions during five hours digestion.

TREATMENTS		HYBRIDS									
		H1	H2	H1	H2	H1	H2	H1	H2	H1	H2
		1 hour		2 hours		3 hours		4 hours		5 hours	
In vitro leaflets	E1	0	0	1.25	2.63	2.0	3.0	0.5	3.0	5.63	3.38
	E2	0	0	4.75	1.13	13.5	1.88	17.88	11.25	27.75	2.8
	E3	0	0	0.75	3.88	3.88	8.5	11.63	18.63	10.25	11.63
	E4	0	1.63	0.13	2.5	6.0	26.25	9.0	21.0	9.38	23.5
	E5	0	0	0.5	0	2.63	0	1.25	0	1.25	0.38
	E6	0	0	0.25	0	0.88	0	0.5	0	1.5	0.13
	E7	0	0	1.25	0	1.25	0	2.63	0.5	3.0	0
	E8	0	0	0.25	0	0.88	0	3.25	0.25	1.75	0.25
In vivo leaflets	E1	0	0	0.13	0	0.13	0	0.25	0	0.63	0
	E2	0	0	1.0	0	0.75	0	2.38	0.10	2.38	1.0
	E3	0	0	0.63	0	0.5	0	0.25	0	1.0	0
	E4	0	0	0	0	0.13	0	0.25	0.15	1.38	1.50
	E5	0	0	0	0	0	0	0	0	0	1.0
	E6	0	0	0	0	0	0	0	0	0	1.0
	E7	0	0	0	0	0	0	0	0	0	0.20
	E8	0	0	0	0	0	0	0	0	0	0.20
Calli	E1-E8	0	0	0	0	0	0	0	0	0	0

H1 = hybrid 1; H2 = hybrid 2; E = enzymatic solution; E1 = 0.5% cellulase R-10, 0.2% macerozyme and 0.1% driselase; E2 = 1.0% cellulase R-10, 0.2% macerozyme and 0.1% driselase; E3 = 1.5% cellulase R-10, 0.2% macerozyme and 0.1% driselase; E4 = 2.0% cellulase R-10, 0.2% macerozyme and 0.1% driselase; E5 = 0.5% cellulase R-10, 1.0% pectinase and 0.5% hemicellulase; E6 = 1.0% cellulase R-10, 1.0% pectinase and 0.5% hemicellulase; E7 = 1.5% cellulase R-10, 1.0% pectinase and 0.5% hemicellulase and E8 = 2.0% cellulase R-10, 1.0% pectinase and 0.5% hemicellulase. Obtained yield x 10^4 protoplasts mL.

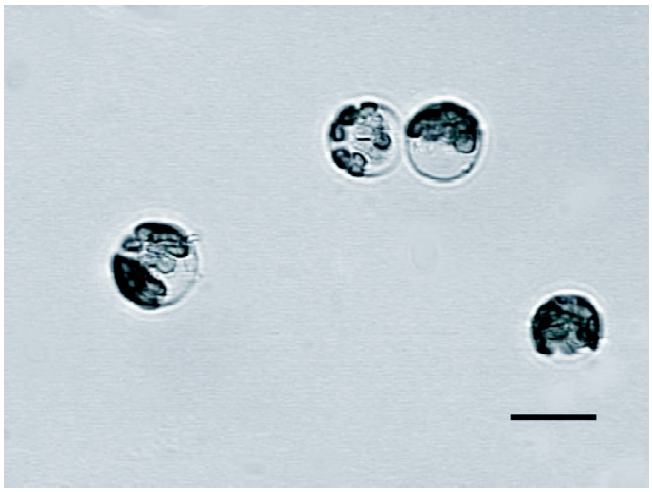

Figure 1 – Viable protoplasts from *in vitro* leaflet tissue of H2 triploid hybrid between napier grass and pearl millet using the enzymatic solution E4. Bar =50 µm.

The maximum protoplast yield for both hybrids was obtained from *in vitro* leaflets: 27.75 x 10^4 protoplasts/mL for H1 hybrid after 5 hours digestion in the enzymatic solution E2 (1.0% cellulase R-10, 0.2% macerozyme and 0.1% driselase), and 26.25 x 10^4 protoplasts/mL for H2 hybrid after 3 hours digestion by the enzymatic solution E4 (2.0% cellulase R-10, 0.2% macerozyme and 0.1% driselase).

Prasertsongskum (2004) also obtained the best result (8.4 x 10^4 protoplasts/mL) with the grass *Vetiveria zizanioides* (L.) Nash when cellulase and macerozyme were present in the enzymatic solution (2.0% cellulase R-10 plus 2.0% macerozyme and 0.5% pectinase).

For both hybrids, the highest efficiency in cell wall digestion was achieved with the combination among cellulase R-10, macerozyme and driselase enzymes. Leaflets from seedlings cultured under *in vitro* conditions were softer, smaller and thinner than those from *in vivo* cultivation, which favored the enzymatic action. These morphological differences were probably due to the lower light intensity and higher humidity under *in vitro* conditions.

Similar results were reported by Komai et al. (1996), who obtained 1.46 x 10^7 protoplasts/g leaves when spinach (*Spinacia oleracea* L.) seedlings cultured *in vitro* were treated from 4 to 10 hours with 2% cellulase R-10 and 0.5% macerozyme R-10. Hu et al. (1999) also used the enzymatic solution of 2% cellulase R-10, 1.0% macerozyme, 0.5% driselase, between 15 and 20 hours, to isolate protoplasts from *in vitro* leaves and hypocotyl of several *Brassica* species.

Although at a small quantity, protoplasts were obtained from leaflets of *in vivo*-cultured seedlings. It is interesting to note, however, that in H1 and H2 hybrids the maximum protoplast yield from both *in vitro* and *in vivo* explants was observed under the same enzymatic treatment. For H1, E2 treatment allowed the production of only 2.38 x 10^4 protoplasts/mL after five hours of digestion, whereas *in vitro* leaflets had a 12-fold higher yield (27.75 x 10^4 protoplasts/mL). As regards H2, the best enzymatic solution was E4, with which 1.5 x 10^4 protoplasts/mL were obtained after five hours digestion of *in vivo* leaflets, whereas only three hours digestion of *in vitro* leaflets were needed to achieve approximately 18-fold higher yield (26.25 x 10^4 protoplasts/mL) (Table 1).

Calli showed not to be a good vegetative material to produce protoplasts from H1 and H2 hybrids.

Vasil et al. (1983) isolated protoplasts from cell suspensions originated from calli of napier grass (*Penisetum purpureum* Schumach.) inflorescences. After extensive work, they concluded that the best enzymatic treatment included 2.5% cellulase R-10, with or without 1.0% macerozyme, which allowed the isolation of 80% protoplasts after six hours digestion.

It must be emphasized that protoplast yield varies significantly in the different studies available in the literature. This is mainly due to the plant material cultivation method and to the production conditions besides other several factors, such as the genotype as well as the enzymatic combinations and concentrations employed in the study.

CONCLUSIONS

Leaflets from seedlings cultured *in vitro* were the best sources to produce protoplasts from napier grass and pearl millet triploid hybrids.

The enzymatic combination among cellulase, macerozyme and driselase led to higher protoplast yield for both studied hybrids.

ACKNOWLEDGEMENTS

The authors wish to thank FAPEMIG for financial support, Capes for Master's scholarship to the first author, CNPq for productivity scholarship, and Prof. Wagner Campos Otoni for his valuable suggestions.

REFERENCES

ABREU, J.C.; DAVIDE, L.C.; PEREIRA, A.V.; BARBOSA, S. Mixoploidia em híbridos de capim-elefante x milheto tratados com agentes antimitóticos. **Pesquisa Agropecuária Brasileira**, Brasília, v.41, n.11, p.1629-1635, 2006.

BARBOSA, S.; DAVIDE, L.C.; PEREIRA, A.V.; ABREU, J.C. Duplicação cromossômica de híbridos triplóides de capim-elefante e milheto. **Bragantia,** Campinas, v.66, n.3, p.365-372, 2007.

FREARSON, E.M.; POWER, J.B.; COCKING, E.C. The isolation, culture and regeneration of *Petunia* leaf protoplast. **Developmental Biology**, Orlando, v.33, p.130-137, 1973.

HANNA, W.W. Melhoramento do capim-elefante. In: PASSOS, L.P.; CARVALHO, L.A.; MARTINS, C.E.; PEREIRA, A.V. (Eds.). **Biologia e manejo do capim-elefante**. Juiz de Fora: Embrapa Gado de Leite, 1999. p.17-28.

HANNA, W.W. Method of reproduction in napiergrass and in the 3X and 6X alloploid hybrids with pearl millet. **Crop Science**, Madison, v.21, p.123-126, 1981.

HANNA, W.W.; GAINES, T.P.; GONZALEZ, B.; MONSON, W.G. Effect of ploidy on yield and quality of pearl millet x napier grass hybrids. **Agronomy Journa**l, Madison, v.76, n.6, p.969-971, 1984.

HU, Q.; ANDERSEN1, S.B.; HANSEN, L.N. Plant regeneration capacity of mesophyll protoplasts from *Brassica napus* and related species. **Plant Cell, Tissue and Organ Culture**, Dordrecht, v.59, p.189–196, 1999.

JAUHAR, P.P.; HANNA, W.W. Cytogenetics and genetics of pearl millet. **Advances Agronomy**, New York, v.64, p.1-26, 1998.

KOMAI, F.; MASUDA, K.; HARADA, T.; OKUSE, I. Plant regeneration from adventitious roots of spinach (*Spinacia oleracea* L.) grown from protoplasts. **Plant Science**, Shannon, n.120, p.89-94, 1996.

MESA-PEREZ, J.M.; CORTEZ, L.A.B.; ROCHA, J.D.; BROSSARD-PEREZ, L.E.; OLIVARES-GOMEZ, E. Unidimensional heat transfer analysis of elephant grass and sugar cane bagasse slow pyrolysis in a fixed bed reactor. **Fuel Processing Technology**, Amsterdam, v.86, p.565–575, 2005.

MURASHIGE, T.; SKOOG, F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. **Physiology Plant**, Bethesda, v.15, p.473-497, 1962.

PASSOS, L.P.; KATTERMAN, F. Produção de calos embriogênicos em capim-elefante, em resposta a diferentes cultivos, explantes e reguladores de crescimento. **Revista da Sociedade Brasileira de Zootecnia**, Viçosa, v.23, n.5, p.743-753, 1994.

PEREIRA, A.V.; VALLE, C.B.; FERREIRA, R.P.; MILES, J.W. Melhoramento de forrageiras tropicais. In: NASS, L.L.; VALOIS, A.C.C.; MELO, I.S.; VALADARES-INGLIS, M.C. **Recursos genéticos e melhoramento**. Rondonópolis: Fundação Mato Grosso, 2001. p.549-602.

PRASERTSONGSKUN, S. Isolation and culture of suspension protoplasts of vetiver. **Songklanakarin Journal of Science and Technology**, Songkhla v.26, n.3, p.411-416, 2004.

SCHANK, S.C.; DIZ, D.A.; WOFFORD, D.S. Seed production on detached culms of pearl millet × elephantgrass hexaploid hybrids. **HortScience**, Alexandria, v.31, n.5, p.827-828, 1996.

SOUZA SOBRINHO, F.; LEDO, F.J.S.; PEREIRA, A.V.; OLIVEIRA, J.S. Avaliação do potencial de propagação por sementes de capim-elefante hexaplóide. **Ciência e Agrotecnologia**, Lavras, v.32, n.3, p.974-977, 2008.

STREZOV, V.; EVANS, T.J.; HAYMAN, C. Thermal conversion of elephant grass (*Pennisetum Purpureum* Schum) to bio-gas, bio-oil and charcoal. **Bioresource Technology**, New York, v.99, n.17, p.8394-8399, 2008.

VASIL, V.; WANG, D.; VASIL, I.K. Plant Regeneration from protoplasts of Napier Grass (*Pennisetum purpureum* Schum.). **Zeitschrift für Pflanzenphysiologie**, Stuttgart, v.111, p.233-239, 1983.

ZENG, S.H.; CHUAN-WU, C.; LUI, H.; JI-HONG, L.; XIU-XIN, D. *In vitro* indução, regeneration and analysis of autotetraploids derived from protoplasts and callus treated with colchicine in *Citrus*. **Plant Cell Tiss Organ Cult**, Netherlands, v.87, p.85-93, 2006.