Induction of Cassava Somatic Embryogenesis in Liquid Medium Associated to Floating Membrane Rafts

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ABSTRACT

*The objective of this study was to examine the effect of two culture systems, liquid medium associated to floating membranes and solid medium, both supplemented with different concentrations of 2,4-D, in the induction of somatic embryogenesis of cassava (*Manihot esculenta *Crantz). Only 28% of the young leaf lobes (with 9 µM 2,4-D) were induced to form organized embryogenic structures (OES) with membrane rafts, compared to 50% of the explants presenting this type of tissue in solid medium with 36 µM of 2,4-D. Despite the lower response observed in liquid medium with membrane, the amount of OES/explant in all 2,4-D concentrations was higher than solid medium. Based on the results and considering the high cost of the membrane rafts, this system was not distinctly superior than solid medium for inducing somatic embryogenesis in cassava.*

Key words: *Manihot esculenta*, Somatic embryogenesis, Membrane rafts, Organized embryogenic structures (OES).

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a tropical perennial shrub cultivated for its starch-containing tuberous roots. It is used as a staple food by more than 500 million people (Roca *et al.*, 1992). Because of its tolerance to adverse environmental conditions, cassava can be cultivated in low fertility soils. Despite its economic importance for tropical countries, until recently, little emphasis has been placed on cassava breeding programs.

Implementation of tissue culture techniques for conventional breeding of cassava is very important to improve its efficiency and speed. Studies on somatic embryogenesis of cassava were first reported by Stamp & Henshaw (1982). Since then, a variety of improvement has been reported, such as the induction of cyclic embryogenesis (Raemakers *et al.*, 1993a), embryo maturation and regeneration of whole plants (Mathews *et al.*, 1993). To date, all reported protocols of cassava somatic embryogenesis induction were established on solid medium. The advantages of solid medium are well known (George, 1993). However, agar-solidified medium might present some undesirable features such as the presence of inhibitory substances (Powell & Uhrig, 1987), low rate of molecules diffusion through the gel matrix (Romberger & Tabor, 1971), water availability (Debergh *et al.*, 1981) and agar nutrient composition that may alter the elemental composition of the medium (Singha *et al.*, 1985). In some cases the use of liquid medium might overcome such problems, providing a more effective selection system as the explant is in direct contact with the culture medium.

Raemakers (1993) demonstrated that in cyclic somatic embryogenesis of cassava, liquid medium produced higher yield of embryos in comparison to solid medium. Recently, Taylor *et al.* (1996) established an embryogenic cell suspension on liquid SH medium (Schenk & Hildebrandt, 1972). Based on this system, the production of the first transgenic cassava plant by particle bombardment was reported (Schöpke *et al.*, 1996). Although Szabados *et al.* (1987) and Stamp and Henshaw (1987) suggested the possibility of somatic embryogenesis induction on liquid medium, conclusive data on this subject were not reported.

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Recently, a new culture system has been successfully used on plant micropropagation programs. In such system, explants were cultured on a flat microporous polypropylene membrane floating in direct contact with the liquid medium. On plant micropropagation studies, this culture system has shown better results when compared to cultures conducted on either liquid and solid medium (Hew *et al.*, 1990; Young *et al.*, 1991; Desamero *et al.*, 1993; Watad *et al.*, 1995). However, it has never been tested in studies on induction and regeneration of somatic embryos.

In the present work, we describe the induction of organized embryogenic structures (OES) in leaf explants of cassava in liquid medium associated to floating membranes in comparison to solid medium. Different concentrations of 2,4-D were tested in both systems.

MATERIALS AND METHODS

In vitro cultured plants of the cassava genotype MCol 22 were obtained from the International Center for Tropical Agriculture (CIAT), Colombia. This genotype is commonly utilized for studies on somatic embryogenesis of cassava. Leaf lobes (3-5 mm of length) from *in vitro* cultured plants were used as explant source, according to Roca *et al.* (1984).

The culture medium consisted of MS salts (Murashige & Skoog, 1962), supplemented with 0.5 mg.L^{-1} thiamine-HCl, 0.5 mg.L^{-1} pyridoxine, 0.5 mg.L^{-1} nicotinic acid, 100 mg.L^{-1} myo-inositol, 2% sucrose and 2.5 g.L^{-1} Phytagel™ as gelling agent for solid medium. 2,4-D was added to the media at different concentrations (9, 18 and 36 µM). The pH was adjusted with KOH to 5.7 and both liquid and solid medium were distributed in 50 ml aliquots into Magenta™ GA-7 vessels (Fig. 1A). The medium was autoclaved for 20 min at 121°C. Cultures were kept in the dark at 28±1°C. After 21 days, cultures were evaluated for the formation of organized embryogenic structures (OES) which were characterized by their smooth surface and highly organized development, and the formation of non-embryogenic friable callus (NEFC) – callus of rapid growth visible in the early stages of culture. The frequency of explants containing OES was also scored.

A completely randomized experimental design was used. Each treatment consisted of 5 replications of 5 explants. OES obtained in both culture systems were transferred to maturation medium, according to Mathews *et al.*, (1993) with minor modifications. The modified medium consisted of MS basal salts supplemented with 0.5 mg.L^{-1} thiamine-HCl, 0.5 mg.L^{-1} pyridoxine, 0.5 mg.L^{-1} nicotinic acid, 100 mg.L^{-1} myo-inositol, 3% sucrose, 2.5g.L^{-1} Phytagel™ and 5 g.L^{-1} active charcoal. Some of the maturated embryos were transferred to germination medium which consisted of MS salts supplemented with 0.5 mg.L^{-1} thiamine-HCl, 0.5 mg.L^{-1} pyridoxine, 0.5 mg.L^{-1} nicotinic acid, 100 mg.L^{-1} myo-inositol, 2% sucrose and 2.5 g.L^{-1} Phytagel™. After 15-20 days of culture, regenerated whole plants were transferred to greenhouse.

RESULTS AND DISCUSSION

Plant micropropagation systems on liquid medium associated to membrane rafts has been successfully used in several works (Watad *et al.*, 1995; Desamero *et al.*, 1993; Adelberg *et al.*, 1992). However, as far as we know this is the first report describing the use of such system for somatic embryogenesis induction.

Although all explants cultivated in both systems tested in this work showed the formation of OES (Fig. 1B and 2), the best response were obtained using solid medium supplemented with 36 µM 2,4-D, where approximately 50% of the explants were able to form embryos (Fig. 2). These results confirmed the data previously reported by Li *et al.* (1998) also using the genotype MCol 22. Similar results were reported by Szabados *et al.* (1987), with the genotype MCol 1505. When leaf explants were cultured on liquid medium associated to membranes rafts, the best results were achieved at 9 µM 2,4-D with 30% of explants with OES. While the number of explants presenting OES was higher in solid medium in all concentrations of 2,4-D (Fig. 2),

the higher degree of OES formation (amount of OES/explant) was observed in liquid medium with low concentrations of 2,4-D (9 and 18 µM) (Table 1). Using liquid medium associated

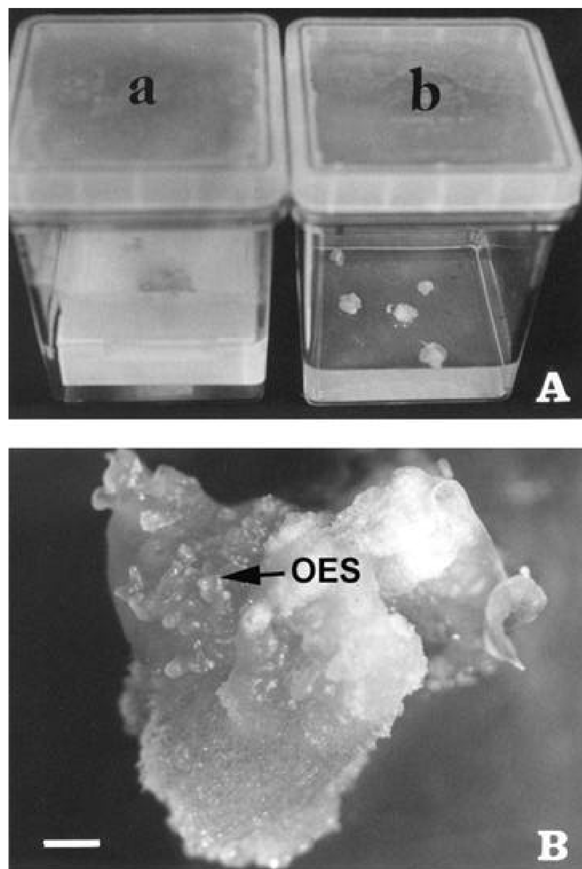
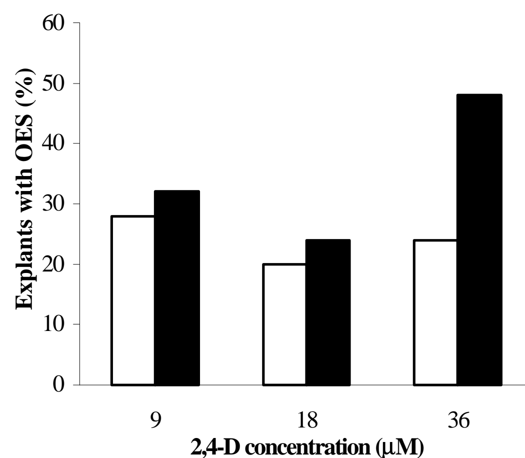


Figure 1. A) Explants after 21 days in liquid medium associated to membrane rafts (a) and in solid medium (b). B) OES obtained in liquid medium (Bar = 2mm).

to membrane rafts for micropropagation of *Aconitum napellus*, Watad *et al.* (1995) demonstrated that higher number of shoots were obtained at low concentration of growth regulators in comparison to agar-solidified medium. It is known that agar gel may restrict the diffusion of large molecules (Romberger & Tabor, 1971) as well as inorganic substances (Faye *et al.*, 1986). Therefore, such response may be due to the better availability of nutrients and growth regulators in the liquid medium.

The use of membrane rafts makes shaking of the cultures unnecessary. Raemakers *et al.* (1993b) showed that after three consecutive cycles of somatic embryogenesis in cassava, the yield of embryos produced in liquid medium was higher



than in solid medium.

Figure 2. Effect of 2,4-D on the frequency of OES formation after 21 days of culture in solid medium (■) and liquid medium/membrane rafts (□).

According to the authors, development of embryos in solid medium could be overcome by the proliferation of callus on the explant. In order to remove the calli, a period of culture in liquid medium under agitation was required. In the present study, the use of membrane rafts for inducing embryogenic cultures reduced proliferation of nonembryogenic callus when compared to solid medium (Table 1).

Table 1. Formation of tissue types from leaf lobe explants with different concentrations of 2,4-D in two culture system, after 21 days.

2,4-D (µM)	Culture medium	Degree of NEFC formation	Degree of OES formation
9	Solid	++	++
	Liquid	+	+++++
18	Solid	+++	++++
	Liquid	++	+++++
36	Solid	+++	+++
	Liquid	++	+++

NEFC: Nonembryogenic friable callus; **OES**: Organized embryogenic structures. Degree of formation assessed from: + = minimum (up to 25% of total tissue produced) to +++++ = maximum (greater than 75% of total tissue produced).

No visible differences were observed between the development of regenerated plants derived

from OES obtained in solid and liquid medium associated to membrane rafts, including embryo maturation and germination stages (data not shown).

According to our results, cultures in liquid medium associated to membrane rafts were suitable for OES induction, specially regarding the amount of OES/explant. However, this system presented some disadvantages in comparison to solid medium. Its initial cost was higher and cleaning of membranes from eventual contamination was difficult and time consuming. When analysed altogether this system, although innovative and suitable for other tissue culture techniques (i.e. micropropagation of some plant species), was not distinctly superior to solid medium for induction of somatic embryogenesis in cassava.

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RESUMO

O objetivo deste estudo foi comparar a indução de embriogênese somática em mandioca (*Manihot esculenta* Crantz) utilizando o sistema de cultivo em meio líquido associado com membranas flutuantes com meio sólido, ambos suplementados com diferentes doses de 2,4-D. Utilizando membranas flutuantes, o melhor resultado foi obtido na concentração de 9 μM 2,4-D, onde apenas 28% dos explantes foliares apresentaram estruturas embriogênicas organizadas (OES). Por outro lado, em explantes cultivados em meio sólido suplementado com 36 μM de 2,4-D a frequência de OES foi de 50%. Embora a frequência de indução embriogênica tenha sido inferior em meio líquido associado com membranas flutuantes, a quantidade de OES por explante foi igual ou superior ao do meio sólido em todas as concentrações de 2,4-D testadas. Baseado nestes resultados, e considerando o elevado custo das membranas, este sistema de cultura não apresentou vantagens significativas para indução de embriogênese somática em mandioca em relação ao meio sólido.

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