Agrotrópica 10(3): 135 -138. 1998
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REGENERATION OF ISOLATED PROTOPLASTS OF *Theobroma cacao*

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Conditions were established for efficient isolation and regeneration of protoplasts of cocoa (*Theobrama cacao*). Protoplasts were obtained from *in vitro* cell suspension, established from callus derived from leaves and buds. The mix cellulase 4% and e macerocyme 2% yielded high number of protoplasts with a high efficiency of isolation and viability. Regeneration of protoplasts in medium containing 7-8% mannitol as osmoticum presented high cell division.

Key words: *Theobroma cacao*, protoplasts, tissue culture

Regeneração de protoplastos de *Theobroma cacao*. Foram estabelecidas condições para o isolamento e regeneração inicial de protoplastos de cacau (*Thebroma cacao*). Protoplastos foram obtidos a partir de células em suspensão oriundas de folhas e gemas. A combinação das enzimas celulase 4% e macerozyme 2% proporcionou um alto número de protoplastos isolados, com uma viabilidade em torno de 80%. O meio de cultura contendo 7-8% de manitol como estabilizador osmótico proporcionou uma eficiente regeneração dos protoplastos com altas divisões de células.

Palavras-chave: *Theobroma cacao*, protoplastos, cultura de tecidos

Introduction

Theobroma cacao is an important crop grown in Brasil and other tropical countries and has been the subject of investigations aimed to establish methods for manipulation in tissue culture ELHAG et al., 1987, Hall and Collin, 1974, Larkin, 1976, Palma and Villalobos, 1989, Thompson et al., 1987, Adu-Ampomah et al., 1988).

Protoplasts are excellent material for a number of experiments on somatic cell genetics and they can be used to evaluate regeneration capabilities of differentiated cells. Protoplasts fusion permits the combinations of unique and desirable cytoplasmic traits, not generally attainable by conventional breeding, because in most crop plants the cytoplasm is inherited only from the maternal parent (Fluhr, 1983). Protoplasts are also an ideal recipient for foreign DNA, providing incorporation and expression issue, such transformation can lead to the production of genetically modified plants.

One of the limitations for genetically manipulation of *Theobroma cacao* is that tissue culture methods are poorly developed. More specifically, few methods have been described in the literature which allows the regeneration of plants originating from protoplasts. The present study reports isolation and regeneration of protoplasts from *T. cacao*.

Received April 28, 1998. Accepted November 27, 1998.

Material and Methods

Plant material

Seeds of *Theobroma cacao* were surface sterilized by briefly dipping in 70% ethanol followed by incubation in a 5% NaOCl solution for 30 min. The seeds were rinsed several times in sterile water. Seeds were sown on sterilized soil and the germination took place in a culture chamber (16th photoperiod, 28°C).

Initiation of callus

Two different media were compared to produce callus from leaves and buds: C1-Murashige and Skoog medium supplemented with 1mg/L cisteine, 1mg/L 2,4 – D, 30g/L sucrose and 8g/L agar and C2 – Murashige and Skoog medium supplemented with 10% coconut water, 1mg/L 2,4 – D, 30 g/L sucrose and 8 g/L agar.

Axillary bud propagation was successfully established using the method described by Flynn et al. (1990). It was desirable to produce plantlets under conditions for the isolation and subsequent regeneration of protoplasts. Callus from the embryogenic axe was obtained on media C2A – Murashige and Skoog medium supplemented with 10% coconut water, 30g/L sucrose and 8 g/L agar and C4 – Murashige and Skoog medium supplemented with 15% coconut water, 1mg/L thiamine, 2 mg/L NAA, 2g/casein hydrolysate, 40g/L sucrose and 8 g/L agar as previously described (Hall and Collin, 1974, Adu-Ampomah et al., 1988).

Single cell suspensions were readily obtained in liquid medium – C2 from leaf callus.

Protoplast isolation and culture

Leaves of two weeks-old seedlings were taken for protoplasts isolation. About 1,0 – 2,0 g leaf material was cut in pieces, plasmolysed for 1h in 6% mannitol and then transferred to 10 mL of different combinations of enzymes. The following enzymes were investigated: driselase (Kyowa Hako), cellulase R-10 (Onozuka, Japan), hemicellulase (Sigma) and macerozyme R-10 (Yakult Pharmaceutical Industry, Japan). Incubation was performed in the dark at 25°C for 24h.

Protoplast were separated from undigested tissue by consecutive filtration and centrifuged for 5 min at 60xg. The pellet was resuspended on Czapeck solution containing 3g/L $NaNO_3$, 1g/L K_2HPO_4, 0.5 g/L Mg $SO_4.7H_2O$, 0.5 g/L KCl and 0.01 g/L $FeSO_4.7H_2O$ with different concentration of mannitol and put on ice. Protoplasts were incubated for 4 days and the proportion of protoplasts that had divided was estimated microscopically. Viability of the protoplasts was determined by a fluorescein diacetate (FDA) test. A stock solution of FDA (1mg/mL acetone) was prepared and stored at 0°C in the dark. Equal volumes of the FDA solution were mixed with a dense protoplast suspension and after 5 min the viability was examined by fluorescence microscopy. Metabolically active protoplasts are visualized by excitation of the accumulated fluorescein with UV light.

Results and Discussion

Callus produced on medium C2 consisted of highly cytoplasmic cells, whereas cells produced on medium C1 were more vacuolated. Medium C2 gave a faster proliferation of yellowish friable callus in comparison to medium C1. It is generally accepted than cytoplasmic cells have a greater potential for regeneration than less cytoplasmic cells. Hence, C2, was chosen as the medium for maintenance of callus originating from leaf material.

Suspension cultures were established from callus derived of leaves and axillary buds. The yield of protoplasts isolated from leaf tissue of *T. cacao* with varying concentration of the enzymes cellulase, hemicellulase and macerozyme is shown in Table 1. The mix, Cellulase 4% and macerozyme 2%, yield higher number of protoplasts. Protoplasts were also isolated from callus material using the enzymes driselase and hemicellulase (Table 2). The isolation efficiency and the viability of protoplasts were high. Of the protoplasts, 80% were viable as judged by FDA staining. These protoplasts were incubated in C2 liquid medium with varying concentration of mannitol, since the osmoticum greatly influence the capability of protoplast to divide in culture. Thompson *et al.* (1987), describing a technique for protoplast isolation, showed that the best results were obtained from young leaves and the protoplasts remained viable and metabolically stable for up to 40h. On the other hand, Leathers and Scragg (1993) developed a method for the preparation of protoplasts from suspension cells. The highest protoplasts yields were obtained using cells harvested during the exponentiall growth phase.

During the first few days, protoplasts gradually increased in volume and sometimes became irregularly shaped.

Division of protoplasts from the suspension culture occurred in medium containing various mannitol concentrations, although that containing 7% and 8% presented high initial divisions (Table 3). When protoplasts were cultured in liquid media containing 5% and 6% mannitol very few divisions were observed during the first 4 days. Protoplasts were incubated in the dark for 7 days and then placed under a photoperiod under stationary conditions. Dividing cell units were viable after two weeks in culture. Figure 1 shows protoplasts regenerating as viewed in epifluorescence microscope using FDA stain.

Table1. Comparisons of enzymes in various combinations for the release of protoplasts from leaf tissue of *T. cacao*.

Enzyme mix			Number of protoplasts
Hemicellulase %	Cellulase %	Macerozyme %	Release per mL
0.0	2	1	1.0×10^2
0.5	2	1	4.0×10^2
0.0	1	1	none
0.0	4	2	3.0×10^4
1.0	4	2	1.5×10^2
0.0	3	1.5	2.0×10^2
1.0	3	1.5	1.0×10^2

Table 2. Enzyme mix utilized for protoplast isolation from cacao callus

Enzymes			Isolations efficiency	Viability of Protoplasts
Cellulase R-10	Hemicellulase	Driselase		
2%	1%	0.5%	85%	65.81 ± 5.82

[1]Mean of five replications

Table 3. Viability of protoplasts after 1 day in the culture and number of division after 4 days in culture on Czapeck medium with different concentration of mannitol.

Mannitol Concentration (%)	Viability of protoplasts[1]	Number of division per mL
5	47.29 ± 2.37	65
6	49.02 ± 3.10	70
6	59.61 ± 2.13	182
8	62.80 ± 3.16	179
10	46.86 ± 1.69	81

[1]Viability is the mean of 10 readings.

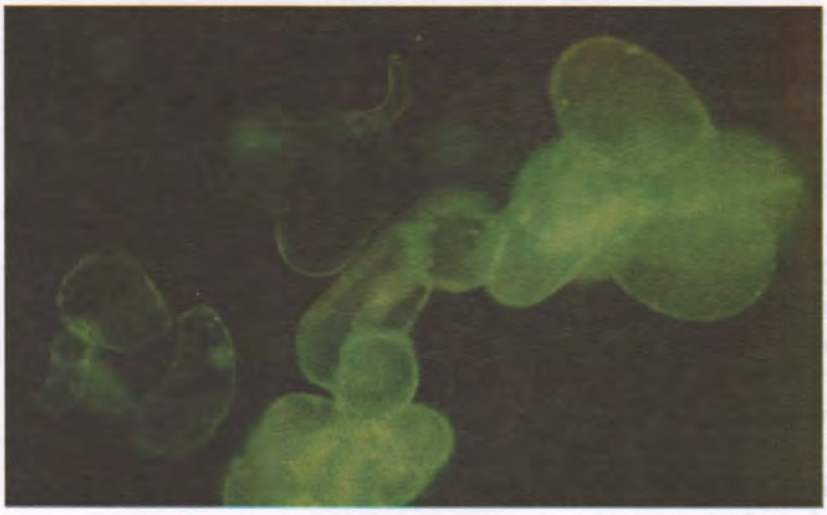

Figure 1. Protoplasts of cocoa regenerating in medium containing 7% mannitol. Cells viewed in epifluorescence microscope using FDA stain.

This work describes a protocol which allows the regeneration of protoplasts from *T. cacao*. Though there have been a few reports in the literature on isolation and culture of protoplasts from *T. cacao*, none has described success in obtaining intact plants. Kanchanapoom and Kanchanapoon (1991) regenerated protoplasts of *Theobroma* in liquid MS medium. The cell wall was regenerated in 10 days and the first division took place in 12-14 days. We have regenerated protoplasts using 7-8% mannitol as osmoticum in the liquid medium. An initial division frequency of more than 50% was obtained in this medium.

Investigation of conditions, such as age of callus and cell suspensions are necessary in order to improve the efficiency of division.

The results described here represent a necessary step towards the genetic modification of *T. cacao* at the protoplasts level.

Acknowledgements

We thank Miss Mara Denise for skillful technical assistance.

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