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Research article

Biochemical precursor effects on the fatty acid production in cell suspension cultures of *Theobroma cacao* L.

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ABSTRACT

Cocoa butter (CB) is composed of 96% palmitic, stearic, oleic, linoleic and linolenic fatty acids that are responsible for the hardness, texture and fusion properties of chocolate. Through *in vitro* plant cell culture it is possible to modify CB lipid profiles and to study the fatty acid biosynthesis pathway on a subcellular level, evaluating fundamental aspects to enhance *in vitro* fatty acid production in a specific and controlled way. In this research, culture media was supplemented with acetate, biotin, pyruvate, bicarbonate and glycerol at three different concentrations and the effects on the biomass production (g/L), cell viability, and fatty acids profile and production was evaluated in *in vitro* cell suspensions culture. It was found that biotin stimulated fatty acid synthesis without altering cell viability and cell growth. It was also evident a change in the lipid profile of cell suspensions, increasing middle and long chain fatty acids proportion, which are unusual to those reported in seeds; thus implying that it is possible to modify lipid profiles according to the treatment used. According to the results of sucrose gradients and enzyme assays performed, it is proposed that cacao cells probably use the pentose phosphate pathway, mitochondria being the key organelle in the carbon flux for the synthesis of reductant power and fatty acid precursors.

1. Introduction

Theobroma cacao L. is a tree from the Amazon region, its fruit contains between 30 and 40 beans with a high fat content, occupying 60% of its dry weight (Tsai and Kinsella, 1982a). Fat from cacao is called cocoa butter (CB), due to its solid state at room temperature. Because of its organoleptic characteristics, it is desirable for the manufacture of several chocolate, cosmetic and pharmaceutical products (Wright et al., 1983). CB is composed of fatty acids organized as symmetrical triglycerides (TAGs), occupying 96% of the total butter content (Fritz et al., 1986; Griffiths and Harwood, 1991). This distribution gives it a special characteristic, a fusion point at 36-37 °C, which makes it more appetizing since this gives CB a creamy consistence and makes it enjoyable to the palate (Lipp and Anklam, 1998).

In cacao, the biosynthetic pathway is not clearly studied, it is not known if precursors are synthesized by the pentose phosphate pathway or the glycolysis pathway (Kruger and Von Schaewen, 2003; Leathers and Scragg, 1993; Reid et al., 1975). Most of the studies about fatty acids in plant models have been conducted on rape, sunflower and soybean seeds, among others (Alonso et al., 2010, 2007; Dieuaide-Noubhani et al., 1995; Grimberg, 2014; Miersch et al., 1986), which contain oils and non solid fats at room temperature as the cacao case, this inspired the development of the current research about the synthesis pathway of CB using an *in vitro* system.

Mature cacao beans contain mainly stearic [18:0] (38,2%) and palmitic acid [16:0] (23,9%) (Saturated) and oleic [18:1] (33,9%) and linoleic [18:2] (3,2%) (unsaturated) (Fritz et al., 1986; Griffiths and Harwood, 1991; Tsai et al., 1982; Tsai and Kinsella, 1981; Wright et al., 1983). Once synthesized, fatty acids are incorporated into a glycerol 3-phosphate backbone through the sequential action of several acetyltransferases to form TAGs (Fritz et al., 1986; Griffiths and Harwood, 1991; Lung and Weselake, 2006; Weselake et al., 1998).

Fatty acid biosynthesis in plants starts with the conversion of sucrose into acetyl-CoA, an intermediary in several metabolic pathways which can also be metabolized from hexose phosphate, free acetate or pyruvate (Möhlmann and Neuhaus, 1997; Murphy,

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1993). Later, it is necessary the malonyl-CoA formation, which serves as a substrate for fatty acid elongation and it is synthesized from acetyl-CoA and bicarbonate thanks to the acetyl-CoA carboxylase enzyme (Lung and Weselake, 2006). This enzyme consists of one or more multifunctional subunits, each one has three active domains with biotin as a cofactor: biotinylated, biotin carboxylase and carboxyl transferase. Fatty acid synthesis continues with the action of a series of ACPases enzymes (acyl carrier proteins) belonging to the Fatty Acid Synthase System (FAS) and that are in charge of stretching the chain through condensation seriated reactions (Lung and Weselake, 2006; Murphy, 1993). Within this enzyme complex there are transacylase, reductase, thioesterase, KAS I-II-III, among others; the final product is palmitoyl-ACP (16:0-ACP). Once the fatty acid chains have been stretched (from 16:0-ACP to 18:0-ACP), desaturase generates double bonds (from 18:0-ACP to 18:1-ACP, etc.) obtaining unsaturated fatty acids (Lung and Weselake, 2006).

In this research, biotechnological and metabolic approaches were used to study the biosynthesis of fatty acids evaluating the effect of different biochemical precursors in fatty acids production using cacao cell cultures. Additionally, a metabolic approach was conducted to hypothesize the pathway of fatty acids in cacao cultures. For this purpose, we evaluated the effect of adding precursors of the fatty acid's biosynthetical pathway in the production of fatty acids in cell cultures of *Theobroma cacao* and the subcellular distribution of cacao fatty acids was estimated, after the addition of the biochemical precursor selected, in different cell organelles.

2. Materials and methods

2.1. Plant material

Cotyledons were taken from mature beans (8 months) of *Theobroma* cacao L. BIOC variety ("Trinitario" hybrid) from commercial crops of Compañía Nacional de Chocolates at San Vicente de Chucurí municipality of Santander department (Colombia), located between 06° 53′00″ N and 73° 24′50″ W.

2.2. Cell suspension culture

Cell suspensions were established from cotyledons of cacao beans according to an enzymatic protocol previously established at the laboratory (Gallego, 2015). Direct establishment of suspensions was made incubating bean explants in liquid media with the pectinase enzyme of a concentration less than 0.1% (US Patent 7,772,002 B1), over twelve days. Later, suspensions were filtered and transferred to a culture medium, which was used as inoculum to establish the suspensions. The culture medium was formulated at the laboratory, CBP (Cacao Biomass Production), based on the nutrient composition of cacao beans from the BIOC variety (Gallego, 2015). The culture conditions were maintained at 79 rpm agitation, 25± 2 °C and 16/8 light/darkness photoperiod. Suspensions from the third subculture (3 weeks after induction) were used for the experiments.

2.3. Biochemical precursors culture

We evaluated four biochemical precursors involved in the *de novo* fatty acid synthesis: biotin (B4639 Sigma-Aldrich, United States), pyruvate (BP356100, Fisher Scientifics, United States) acetate (S5636, Sigma-Aldrich, United States) and bicarbonate (S631, Fisher Scientifics, United States). Glycerol (G2025, Sigma-Aldrich, United States) which is involved in the triglycerides assembly was also included. The five precursors were evaluated at three concentration levels (high, medium, low): 10, 50 and 100 ppm respectively. Glycerol was evaluated at 3, 6 and 12% due to its viscosity. This assay was carried out using a microplate system developed by the work team. The system consisted of plastic containers holding 6 wells microplates, each one with 5 ml capacity with an external filter disk attached to the microplates for air and humidity quality control (Gallego, 2015). For each experiment 4 ml of suspension per well was used. Each container represented a replicate and each well represented a repetition. The system was incubated over 12 days at 79 rpm, 25+ 2 °C.

2.4. Analytical methods

As a growth measure for cell suspensions the packaged cell volume (PCV), described by Mustafa et al. (2011) was determined. To evaluate cell viability 5% fluorescein diacetate [FDA] (F7378, Sigma-Aldrich) was used and optical observation was performed using an Eclipse Nikon 80i microscope, adapted to a Nikon DS-Fi1 digital camera and a C-HGR1 fluorescence lamp (Nikon, Japan).

2.4.1. Statistical design and analysis

A completely random factorial design with two factors was made: biochemical precursor (pyruvate, acetate, bicarbonate, biotin and glycerol) and concentration level (high, medium, low). Data was analyzed using the statistical software *R* version 2.14.1 with a confidence level of 95%.

2.5. Protoplasts and organelles obtained from cells in suspensions

The protocol used for obtaining protoplasts was modified from Leathers and Scragg (1993). 600 μl was taken from a suspension with 9–10 days of growth to which 100 ppm biotin was previously added, it was transferred into an enzymatic mix (2 ml of 2% Macerozime and 1.5% Celulase) (M481 and C224, PhytoTechnology Laboratories, United States) for 20 h at 26 °C. Afterwards, it was filtered through a nylon membrane (45 μm), retained cells were washed with digestion buffer (D-sorbitol [494 mM], MES Buffer pH 5.5 [2,6 mM]). Then, they were centrifuged (HITACHI CR22N centrifuge) at 510×*g* for 4 min at 4 °C. Supernatants were discarded and 5 ml of wash buffer was added (D-sorbitol [505 mM], potassium chloride [200 mM], HEPES sodium salt pH 7.5 [5 mM] and anhydrous calcium chloride [1.4 mM]). Centrifugation was performed two more times. Pellet was re-suspended on a new plastic tube with 6 ml of lysis buffer (D-sorbitol [350 mM], Tris pH 8.0 [100 mM], EDTA Na_2 [1 mM], Mercaptoethanol [0.3% v/v]); cells were softly filtered using a 21-gauge needle five times, aiming to break cell membranes and releasing organelles, which were then incubated on ice for 20 min. Samples were centrifuged at 510×*g* for 5 min at 4 °C. On the pellet there were cell wall residues, membranes and nuclei, so the supernatants were used (in which the organelles and cytoplasmic content remained). These supernatants were centrifuged later for 20 min at 29.200×*g* aiming to deposit organelles at the bottom of the tube; then they were incubated in 600 μl of resuspension buffer (D-sorbitol [500 mM], Tris pH 8.0 [100 mM], EDTA Na2 [1 mM], Mercaptoethanol [0.3% v/v]). Samples were observed on the microscope with 0.1% calcofluor white. Absence of stain on the cells was indicative of lacking a cell wall.

2.6. Organelle isolation

Sucrose concentration gradients used to separate each organelle were defined according to the report by (Hájek et al., 2004; Leathers and Scragg, 1993; Wiley Interscience, 2009). Gradients were prepared using commercial sugar, TES-Mg buffer pH 7.5 (Tris pH 8.0 [10 mM], EDTA Na2 [1 mM], sodium chloride [100 mM], and magnesium chloride [1 mM]); the refraction index was measured