Effect of temperature on polyunsaturated fatty acid accumulation in soybean seeds

Anna Cristina Lanna[1], Inês Chamel José[1,2], Maria Goreti de Almeida Oliveira[1,3], Everaldo Gonçalves Barros[1,4] and Maurilio Alves Moreira[1,3,*]

*[1] Instituto de Biotecnologia Aplicada à Agropecuária (BIOAGRO), Universidade Federal de Viçosa, CEP 36570-000, Viçosa, MG, Brasil (Present address: Embrapa Arroz e Feijão, Rodovia Goiânia - Nova Veneza, Km 12, CEP 75375-000, Santo Antônio de Goiás, GO, Brasil); [2] Departamento de Tecnologia de Alimentos, Universidade Federal de Viçosa, CEP 36570-000, Viçosa, MG, Brasil; [3] Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Viçosa, CEP 36570-000, Viçosa, MG, Brazil; [4] Departamento de Biologia Geral, Universidade Federal de Viçosa, CEP 36570-000, Viçosa, MG, Brasil. * Corresponding author: moreira@ufv.br*

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Soybean oil contains around 60 % of polyunsaturated fatty acids, which are responsible for the low oxidative stability of soy-derived products. Soybean lines with low linolenic acid content can be obtained by genetic manipulation; however, a high proportion of the variation in fatty acids content is due to environmental factors. This work aimed to determine the effect of temperature on oil composition and on the activity of the enzymes CDP-choline:1,2–diacylglycerolcholine phosphotransferase (CPT) and acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT), responsible for maintenance of polyunsaturated fatty acids in the cytoplasmic acyl-CoA pool, that is used for oil synthesis in the seeds. CAC-1, a soybean variety with linolenic acid content of about 8 % and CC4, a BC_3F_4 CAC-1 derived line, with about 4 % linolenic acid, were used. The lines were cultivated under two temperature: 34/28ºC or 22/13ºC - day/night. The seeds were collected along seven development stages, according to their fresh weight. Fatty acid analysis was carried out by gas chromatography and CPT and LPCAT activities were determined by measuring the radioactivity incorporated in their products, phosphatidyl-[^{14}C]choline and phosphatidylcholine-[^{14}C]oleoyl, respectively. Linolenic acid contents were 3.89 and 6.92 % for line CC4 and 7.39 and 12.49 % for variety CAC-1, when submitted to high and low temperature conditions, respectively. Both enzymes were more active, in the development stages analyzed, in seeds produced under low temperature. Kinetics characterization of CPT and LPCAT were conducted previously.
Key words: Cholinephosphotransferase, kinetic characterization, linoleic acid, linolenic acid, lysophosphatidylcholine acyltransferase, soybean seed.

Efeito do acúmulo de ácidos graxos poliinsaturados em sementes de soja: O óleo de soja contém cerca de 60% de ácidos graxos poliinsaturados, que são responsáveis pela baixa estabilidade oxidativa de produtos derivados. Linhagens de soja com baixo conteúdo de ácido linolênico têm sido obtidas por meio de manipulações genéticas; no entanto, alta proporção da variação no conteúdo de ácidos graxos do óleo de soja é devida a efeitos ambientais. Este trabalho objetivou determinar o efeito da temperatura ambiente na composição do óleo e na atividade das enzimas CDP-colina: 1,2 – diacilglicerolcolina fosfotransferase (CPT) e acil-CoA: lisofosfatidilcolina aciltransferase (LPCAT), responsáveis pela manutenção do pool de acil-CoA citoplasmático, que é usado na síntese do óleo nas sementes. Neste trabalho usou-se a variedade de soja CAC-1, que contém cerca de 8 % de ácido linolênico, e CC4, linhagem RC_3F_4 derivada de CAC-1, com cerca de 3 % de ácido linolênico. A variedade e a linhagem foram cultivadas em casa de vegetação, em dois ambientes de temperatura: 34/28ºC e 22/13ºC - diurna/noturna. As sementes foram coletadas ao longo de sete estádios, de acordo com a massa fresca. A análise de ácidos graxos foi efetivada por cromatografia gasosa e, as atividades de CPT e LPCAT, foram determinadas por meio da medida da radioatividade incorporada em seus produtos, fosfatidil-[^{14}C]colina e fosfatidilcolina-[^{14}C]oleoil respectivamente. Os conteúdos de ácido linolênico foram 3,89 e 6,92 % para a linhagem CC4, e 7,39 e 12,49 % para a variedade CAC-1, quando cultivadas sob condições de alta e baixa temperatura, respectivamente. Ambas as enzimas foram mais ativas, nos estádios de desenvolvimento analisados, em sementes produzidas em condições de baixa temperatura. As caracterizações cinéticas de CPT e LPCAT foram conduzidas previamente.
Palavras-chave: ácido linolênico, caracterização cinética, colinafosfotransferase, lisofosfatidilcolina aciltransferase, sementes de soja.

Abbreviations: CoA – coenzyme A, CPT – cholinephosphotransferase, DAG – diacylglycerol, DAGAT – diacylglycerol acyltransferase, LPCAT – lysophosphatidylcholine acyltransferase, PC – phosphatidylcholine, TAG – triacylglycerol.

INTRODUCTION

Soybean seeds contain about 21 % oil, 40 % protein, 34 % carbohydrates and 5 % ash (Burton, 1997), although cultivars with less than 18 % oil or over 50 % protein may be found (Yadav, 1996).

The oil fraction is essentially composed of triacylglycerols, and the composition and distribution of fatty acids in the triacylglycerol molecules determine the oil quality, its nutritional value, flavor and physical properties, such as oxidative stability and melting point. Soybean oil, like most edible oils, is composed of palmitic (C16: 0), stearic (C 18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) acids. Oleic, linoleic and linolenic acids are 18 carbon unsaturated fatty acids, containing one, two and three cis double bonds interrupted by a methylene group, respectively. The double bond positions in the acyl chain from the carboxyl terminal are δ-9 in C18:1, δ-9 and 12 (or ω-6, counting from the methyl terminal) in C18:2, and δ -9 - 12 and –15 (or ω-3) in C18:3. Oleic acid is also referred to as monounsaturated fatty acid, while the linoleic and linolenic acids as polyunsaturated fatty acids (Yadav, 1996).

Soybean oil contains about 11 % palmitic, 4 % stearic, 24 % oleic, 54 % linoleic and 7 % linolenic acids (Kinney, 1996). The quality of the oil fraction varies considerably among these sources and it depends on the fatty acid composition and, specially, on the proportion of unsaturated fatty acids, mainly oleic, linoleic and linolenic acids (Somerville and Browse, 1991). Due to high levels of polyunsaturated fatty acids the quality of soybean oil is not ideal for industrial purposes, mainly due to its low oxidative stability. Currently, chemical hydrogenation is the industrial process used to increase the oxidative stability of the soybean oil (Hildebrand and Collins, 1998). However, this process also generates significant amounts of trans fatty acids, which have been related with heart problems in animals and humans (Yadav, 1996). For this reason, there is a considerable interest on the genetic modification of soybean oil composition, by traditional breeding or by the use of molecular biology techniques. These modifications could avoid the production of the undesirable trans fatty acids and also produce oils with better nutritional and functional attributes (Wang and Hildebrand, 1988; Osório et al., 1995; Kinney, 1996).

Most of the polyunsaturated fatty acids production in developing seeds, occurs via desaturation of oleic and linoleic acids catalyzed by desaturases in the smooth endoplasmatic reticulum (Ohlrogge et al., 1991; Somerville and Browse, 1991; Arondel et al., 1992). The substrate for the desaturases is PC (Slack et al., 1979; Browse and Slack, 1981). The fatty acids linked to PC, which may become unsaturated, can subsequently be incorporated into storage triacylglycerol (TAG) molecules. The linoleic and linolenic acid levels in the oil depend on their biosynthesis rate and availabilities (Yadav, 1996). The polyunsaturated fatty acids can become available through two distinct mechanisms: (a) reversible reaction by which cholinephosphotransferase (CPT, CDP-choline: 1,2–diacylglycerolcholine phosphotransferase, EC 2.7.8.2) converts phosphatidylcholine (PC) containing polyunsaturated fatty acids into diacylglycerols (DAGs), which can be used for oil synthesis via diacylglycerol acyltransferase (DAGAT) (Slack et al., 1983; Stymne and Stobart, 1984) and (b) reversible reaction by which acyl-CoA: lysophosphatidylcholine acyltransferase (LPCAT, EC 2.3.1.23) catalyses the exchange of acyl groups, generally between oleoyl-CoA and a polyunsaturated acyl group linked to position 2 of PC (Stymne et al., 1983; Griffiths et al., 1988).

CPT catalyzes the reversible exchange between the PC pool and DAGs. Due to reaction reversibility, PC can act as a precursor of highly unsaturated molecular species of DAGs in seeds that accumulate polyunsaturated fatty acids in the oil fraction. It is a key enzyme in the metabolism of oilseeds, thus its activity and regulation mechanisms are essential for understanding the fatty acid distribution for lipid synthesis (Vogel and Browse, 1995).

LPCAT catalyzes the reversible reaction between acyl groups of acyl-CoA cytoplasmatic pool and unsaturated acyl group linked to position 2 of PC. The reaction equilibrium is towards PC synthesis, i.e., lysophosphatidylcholine acylation, since it results in cleavage of an energy-rich thioester bond (Stymne and Stobart, 1984). According to Griffiths et al. (1988) the permutation between acyl groups is dictated by: (a) velocity and specificity of exchange of acyl groups between acyl-CoA and PC; (b) unsaturation rate of fatty acids in PC and (c) activity and specificity of LPCAT.

According to Stymne et al. (1983) oleoyl-CoA was a preferential substrate in the acyl-CoA pool for LPCAT activity while stearyl-CoA was completely excluded. Griffiths et al. (1985) and Stobart and Stymne (1985) showed that during exchange of acyl groups, oleoyl-CoA enters position 2 of PC, liberating linoleate which is preferentially used in the acylation of position 2 of glycerol 3-phosphate. Thus, this enzyme regulates the type of acyl groups constituting the TAGs, which accumulate in developing seeds. According to Stobart et al. (1983), the exchange of acyl groups between acyl-CoA and PC is a major step for regulating quality of

polyunsaturated fatty acids in the acyl-CoA pool for oil synthesis in developing safflower seeds.

The soybean breeding program developed at the Biotechnology Institute of the Federal University of Viçosa aims to obtain special soybean lines for the food industry, and has created a germplasm which includes lines with low linolenic acid content (Moreira, 1999). In this work, near isogenic soybean lines, contrasting for linolenic acid content, which were developed in this breeding program, were used to investigate the effect of temperature on soybean oil composition and on the activities of the enzymes CPT and LPCAT during seed development, after their kinetic characterization in the soybean seeds.

MATERIAL AND METHODS

Genetic material: Soybean (Glycine max (L.) Merrill) commercial cultivar CAC-1 and the CAC-1-derived line CC4, were used in this experiment. CAC-1 has a normal linolenic acid content (about 8 %), while CC4 has a low linolenic acid content (about 3 %).

The CC4 line was obtained by crossing CAC-1 with BARC-12 (with less than 3 % of linolenic acid), a soybean line developed by the USDA-ARS in Beltsville, Maryland USA. F1 plants were selfed, and the F2 seeds selected for their low linolenic acid content, were sown in a greenhouse and the corresponding F2 plants were backcrossed with CAC-1. This procedure was followed by selfing and seed selection was conducted until the third backcross cycle to produce the BC_3F_4 CC4 line, which presented low linolenic acid content.

The plants were cultivated from May to September 2000. Until the beginning of the reproductive period, they were grown under an average daily temperature of 31ºC and then transferred to two distinct greenhouses: one with a ventilation system, with average maximum and minimum temperatures of 22ºC and 13ºC, respectively; the other with an artificial heating system which kept the average maximum and minimum temperatures at 34 ºC and 28 ºC, respectively. The temperatures were measured daily at 5:00 pm.

For each temperature condition, 45 pots were planted with each genotype, three plants per pot. The experiment was arranged in a completely randomized design.

The seeds were harvested in seven development stages, based on the fresh weight of the seed: 1 - 0 to 75 mg; 2 - 76 to 150 mg; 3 - 151 to 225 mg; 4 - 266 to 300 mg; 5 - 301 to 375 mg; 6 - 376 to 450 mg; and 7 - mature seed. Immediately after harvest and weighing, the seeds were frozen in liquid nitrogen and stored at -80ºC for subsequent analyses. At least 100 seeds were collected for each stage.

Oil and fatty acid determinations were carried out in each stage and the activities of the enzymes CPT and LPCAT were determined until the 6th development stage.

Determination of total oil content: Total oil in the seeds was determined in a Soxhlet extractor, using petroleum ether as solvent, according to the procedure described in the Analytical Rules of the Adolfo Lutz Institute (1985), with a 24 h-reflux. For each development stage three samples (4 g each) were extracted.

Determination of fatty acid content: The fatty acid content in soybean seed oil fraction was determined by gas chromatography. The seeds were lyophilized and ground to a fine powder and approximately 150 mg of the powder was placed in a 1.5 mL microtube. One mL of hexane was added to each sample and the mixture was maintained at 4ºC for about 16 h under an N_2 atmosphere. Following that period the lipid fraction was poured into culture tubes and the solvent evaporated by N_2 bubbling. In order to obtain methyl esters, the methodology described by Jham et al. (1982) was used. Following derivatization, 1 µL aliquots were injected in a CG-17A gas chromatograph equipped with an auto sampler (Shimadzu, model AOC-17) and an integrator (Shimadzu, model C-R7A). The Carbowax capillary column (30m x 0.32mm) was maintained at 225 ºC, with the injector and detector temperatures being 245ºC and 280ºC, respectively. The carrier gas was nitrogen, at a flow rate of 1.1 mL.min^{-1}.

Enzymatic extract: Approximately four grams of soybean seeds were homogenized in a blender for 2 min in 5 mL of 0.02 mol.L^{-1} Tris-HCl (pH 7.5), containing 1 x 10^{-3} mol.L^{-1} EDTA, 1 x 10^{-3} mol.L^{-1} EGTA, 5 x 10^{-3} mol.L^{-1} DTT and 0.33 mol.L^{-1} sorbitol. The homogenate was filtered through two gauze layers. This procedure was repeated twice with the residue, and the filtrates were combined. The filtrate was centrifuged at 18,000 x g_n for 10 min, at 4 ºC, and the supernatant was transferred to an ultracentrifuge tube and spun at 105,000 g_n for 1 h, at 4ºC. The pellet (microsomal fraction) was resuspended in 8 ml 0.01 mol.L^{-1} Hepes-KOH (pH 7.5), containing 2 x 10^{-3} mol.L^{-1} EDTA, 5 x 10^{-4} mol.L^{-1} EGTA and 1 x 10^{-3} mol.L^{-1} DTT, and centrifuged again at 105,000 g_n for 1 h, at 4ºC. The washed microsomes were resuspended in 4 mL 0.01 mol.L^{-1} Hepes-KOH buffer (pH 7.5), containing 2 x 10^{-3} mol.L^{-1} EDTA and 5 x 10^{-4} mol.L^{-1}

EGTA. Throughout the whole procedure, the samples were kept in an ice bath. The microsomal fraction was divided into 0.5-mL-aliquots and immediately frozen at -80ºC (Slack et al., 1979; Slack et al., 1985; Vogel and Browse, 1995).

Determination of total proteins in the microsomal extract: The determination of protein concentration was done by the bicinchoninic acid method (Smith et al., 1985), using bovine serum albumin (BSA) as standard. To 25 µL aliquots of the sample 1 mL of the stock reagent (bicinchoninic acid/copper sulphate solution, 2/1, v/v) was added. The mixture was stirred and placed in a water bath at 37ºC for 30 min. The tubes were then cooled at room temperature and the absorbance read at 562 nm.

Radioactive compounds and radioactivity determination in samples containing ^{14}C: Cytidine 5'-diphospho[metyl-^{14}C]choline and [1-^{14}C]oleoyl-CoA were purchased from Amersham Pharmacia Biotech. The specific activities were 2.07 GB.mmol^{-1} (98 % radiochemical purity) and 2.07 GB.mmol^{-1} (99 % radiochemical purity), respectively. The stock solutions were prepared in Milli-Q water and stored at -20ºC. The radioactivity in the test solutions was determined in a LS 6500 model liquid scintillation spectrometer from Beckman. Radioactivity was measured for 15 min, twice for each sample, or until the sigma error was below 1 %. The scintillation cocktail was purchased from Sigma Company. In all experiments, the radioactivity detected in cpm (counts per minute) was transformed in nmoles of CDP-[^{14}C]choline incorporated into diacylglycerols.min^{-1} per mg of microsomal protein for CPT and nmoles of [^{14}C]oleoyl-CoA incorporated into lysophosphatidylcholine-palmitoyl.min^{-1} per mg of microsomal protein for the LPCAT.

Determination of cholinephosphotransferase activity through the incorporation of CDP-[^{14}C]choline into diacylglycerols: To analyze the activity of CPT on CDP-choline (tracer CDP-[^{14}C]choline), aliquots of microsomal extract, corresponding to 0.1 mg of protein, were added along with 0.06 mol.L^{-1} Tricine-KOH buffer (pH 8.0), containing 15 x 10^{-3} mol.L^{-1} $MgCI_2$, 1 x 10^{-3} mol.L^{-1} EDTA, 5 x 10^{-4} mol.L^{-1} EGTA and 5 x 10^{-3} mol.L^{-1} DTT, 10 µL of CDP-choline 4 x 10^{-5} mol.L^{-1} and 10 µl of CDP-[^{14}C]choline 4x10^{-7} mol.L^{-1} (0,14 MBq). The reaction mixture (150 µL) was incubated at 30ºC and, after 5 min, the reaction was stopped by adding 0.35 mL 0.1 mol.L^{-1} HCl. The lipid extraction was performed by adding 1.0 mL of a chloroform/methanol mixture (1/1, v/v) and the total radioactivity of the organic phase was determined in liquid scintillation spectrometer (Slack et al., 1985).

Determination of the lysophosphatidylcholine acyltrans-ferase activity through the incorporation of [^{14}C]oleoyl-CoA in lyso-phosphatidylcholine: To determine the activity of the LPCAT, an aliquot of the microsomal extract (equivalent to 0.12 mg of protein) was mixed with 0.1 mol.L^{-1} potassium phosphate buffer (pH 7.2), containing 1 x 10^{-4} mol.L^{-1} lysophosphatidylcholine palmitoyl; 50 µL of 1.6x10^{-4} mol.L^{-1} oleoyl-CoA and 200 µL of 3.4 x 10^{-6} mol.L^{-1} [^{14}C]oleoyl-CoA (0,7 MBq). The reaction mixture (1 mL) was incubated at 30ºC and after 5 min and was ended by addition of 3 mL 0.15 mol.L^{-1} acetic acid. Lipid extraction was performed by adding 4 mL of a chloroform/methanol/acetic acid mixture (10/20/7.5, v/v/v). Following the organic phase separation, 20 mL of chloroform/water mixture (1/1, v/v) were added and the organic phase was again separated, evaporated by bubbling N_2 and resuspended in 0.5 mL of chloroform. Finally, the organic phase total radioactivity was determined in the liquid scintillation spectrometer (Stymne and Stobart, 1984).

Enzyme kinetic characterization: Soybean seeds of the commercial cultivar CAC-1 were used. The plants were cultivated in the greenhouse from May to September 2000, in a completely randomized design. Twenty pots were planted, with three plants per pot. Seeds with fresh weight of approximately 300 mg were harvested, frozen in liquid nitrogen and stored at -80ºC for subsequent analyses.

Time curve determination for the formation of phosphatidyl-choline catalyzed by cholinephosphotransferase: CPT activity was analyzed at 2.5; 5; 10; 15; 20; and 25 min, using 80.5 µL of microsomal extract; 10 µL 4 x 10^{-5} mol.L^{-1} CDP-choline and 10 µL 4 x 10^{-7} mol.L^{-1} (0.14 MBq) CDP-[^{14}C]choline (tracer).

Temperature curve for the formation of phosphatidyl-choline catalyzed by cholinephosphotransferase: The effect of temperature on rate of phosphatidyl-choline formation, catalyzed by CPT, was determined at 5, 10, 15, 20, 25, 30, 35 and 40ºC, using 85.1 µL of microsomal extract; 10 µL 4 x 10^{-5} mol.L^{-1} CDP-choline and 10 µL 4 x 10^{-7} mmol.L^{-1} (0.14 MBq) CDP-[^{14}C]choline (tracer) and a reaction time of five minutes.

Determination of kinetic parameters Km_{app} and $Vmax_{app}$ for cholinephosphotransferase: The kinetic parameters of

CPT was determined by using CDP-choline as substrate, at the concentrations of 0.25; 0.5; 1; 2; 4; 8; 16; 32; 64 and 128 x 10^{-5} mol.L^{-1}, in addition to CDP-[^{14}C]choline (tracer) at the concentration of 4 x 10^{-7} mol.L^{-1} (MBq). The kinetic parameters, in the stationary state, were obtained through non-linear regression analysis, using the computer program Enzifitter (Leatherbarrow, 1987).

Time curve for the formation of phosphatidylcholine-oleoyl catalised by lysophosphatidylcholine acyltransferase: The LPCAT activity was analyzed at 2.5; 5; 10; 15; 20; and 25 min, using 77.4 µL of the microsomal extract; 50 µL 1.6 x 10^{-4} mol.L^{-1} oleoyl-CoA and 200 µL 3.4 x 10^{-6} mol.L^{-1} (0.7 MBq) [^{14}C]oleoyl-CoA (tracer).

Temperature curve on phosphatidylcholine-oleoyl formation catalyzed by lysophosphatidylcholine acyltransferase: The effect of temperature on the formation of phosphatidylcholine-oleoyl, catalyzed by LPCAT, was determined at 5, 10, 15, 20, 25, 30 e 35°C, using 92.3 µL of the microsomal extract; 50 µL 1.6 x 10^{-4} mol.L^{-1} oleoyl-CoA and 200 µL 3.4 x 10^{-6} mol.L^{-1} (MBq) [^{14}C]oleoyl-CoA (tracer) in a reaction time of 5 min.

Determination of kinetic parameters Km_{app} and $Vmax_{app}$ for lysophosphatidylcholine acyltransferase: The LPCAT kinetic parameters were determined by using oleoyl-CoA as substrate, at concentrations of 2; 4; 8; 16; 49; 64 and 1600 x 10^{-5} mol.L^{-1}, in addition to [^{14}C]oleoyl-CoA (tracer) at concentrations of 3.4 x 10^{-6} mmol.L^{-1} (0.7 MBq). The kinetic parameters in the stationary phase were obtained using non-linear regression, utilizing the computer program Enzifitter (Leatherbarrow, 1987).

RESULTS AND DISCUSSION

Effect of temperature on polyunsaturated fatty acid accumulation and on the activity of the enzymes CDP-choline: 1, 2 – diacylglycerolcholine phosphotransferase and acyl-CoA: lysophosphatidylcholine acyltransferase during development of soybean seeds: The soybean oil composition, particularly in relation to the unsaturated fatty acids, was markedly different when the CC4 and CAC-1 plants were grown under contrasting temperatures. These data are shown in figure 1 (A, B, C and D), where palmitic and stearic acid contents were not affected by the temperature in the range tested. On the other hand, in both cultivars grown under low temperatures, oleic acid content decreased while linoleic and linolenic acid contents increased. Oleic acid reached, on average, 43 % of the seed fatty acid pool for both cultivars grown under high temperature, while the average content of this fatty acid was 20 % in seeds grown under low temperatures. The average contents of linoleic and linolenic acids were 38 % and 6 % under high temperatures and 57 % and 10 % under low temperatures, respectively.

The temperature had an effect on the fatty acid composition of the oil fraction in both genotypes and the fatty acid contents vary considerably among the various stages of seed development. Table 1 contain the variance analysis, in which the effects of temperature, seed genotype and development stage on soybean oil composition were determined. According to Wilcox and Cavins (1992) the enzymes controlling fatty acid biosynthesis in soybean seeds are responsive to the temperature during pod filling. However, the enzymes involved on this process and the mechanism of their temperature-induced regulation are still under investigation.

Searching the enzymatic pathways associated with the enrichment of polyunsaturated fatty acids in acyl-CoA

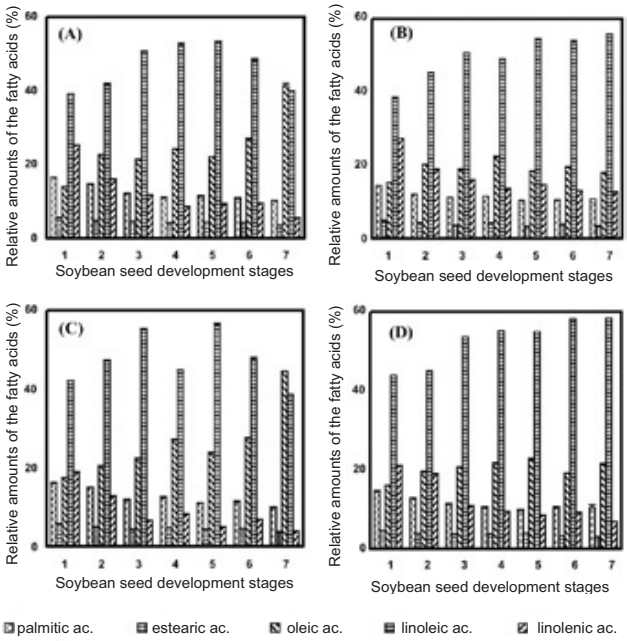

Figure 1. Relative amounts of the five fatty acids present in the soybean oil of commercial variety CAC-1 and CC4 line grown under high (A and C) and low (B and D) temperature conditions, respectively. The soybean seed development stages were defined according to seed fresh weight: 1 - 0 to 75 mg, 2 - 76 to 150 mg, 3 - 151 to 225 mg, 4 - 226 to 300 mg, 5 - 301 to 375 mg, 6 - 376 to 450 mg and 7 - mature seed.

Table 1. Values and significances of square means, means and coefficients of experimental variation obtained from a completely randomized design variance analyses, using a soybean commercial variety CAC-1, and a CAC-1 derived line (CC4), for the palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) acid contents.

Source of deviation	Degrees of freedom	Square Means				
		C16:0	C18:0	C18:1	C18:2	C18:3
DS	6	11.07**	3.74**	278.08**	281.28**	354.87**
T	1	42.30**	9.50**	867.04**	407.05**	285.84**
DS*T	6	20.75**	0.10**	188.81**	147.80**	2.80**
G	1	2.9^{ns}	0.0082^{ns}	81.02**	101.64**	351.35**
DS*G	6	0.06**	0.088**	9.77**	7.62**	10.73**
T*G	1	0.29**	0.087^{*}	3.88**	19.82**	2.98**
DS*T*G	6	0.21**	0.39**	9.80**	30.87**	4.78**
Residue	56	0.77	0.013	0.040	0.060	0.068
Mean		11.89	4.11	22.51	48.98	12.50
CV (%)		1.20	2.72	0.88	0.49	2.08

[1] DS development stage, T – temperature, G – seed genotype
** Significant at 1% probability, by the F test
*Significant at 5% probability, by the F test
[ns] Non –significant

cytoplasm pool, the activities of CPT and LPCAT were determined in various development stages of CAC-1 and CC4 cultivated at high and low temperatures.

The data for CPT are shown in figure 2 (A and B), in which can be observed higher activity in plants grown under low temperature conditions, both in CAC-1 and CC4, in development stages prior to physiological maturity, i.e., throughout pod filling. According to Slack et al. (1985), CPT activity in oil seeds is significantly induced during seed development as triacylglycerols accumulate, and according to Cheesebrough (1989), CPT activity in soybean seeds is regulated by the temperature during seed development. This author observed an increased in CPT activity in plants grown at 20ºC compared to plants grown at 35ºC.

The data for LPCAT are shown in figure 3 (A and B). In both genotypes, the LPCAT activity was higher in seeds grown under low temperature conditions. This enzyme showed greater activity in the initial stages of seed development, when linolenic acid production is also higher. According to Wang and Hildebrand (1988), linolenic acid production is regulated in developing soybean seeds throughout pod filling, being higher in early stages of seed formation, with its relative amount decreasing in the final development stages.

These results showed that CPT and LPCAT were more active under low temperature conditions. Our work demonstrated that the soybean plant responds to temperature decrease by increasing the level of polyunsaturated fatty acids in response to a rise of cholinephosphotransferase and lysophosphatidylcholine acyltransferase activities. The enzyme cholinephosphotransferase is also involved in the production of structural lipids of biological membranes. Therefore, our results indicate the involvement of these enzymes in controlling the pool of cytoplasm polyunsaturated fatty acids for lipid membrane biosynthesis enhancing the tolerance of the soybean plant to cold temperatures.

Enzyme kinetic characterization: Figure 4 presents the time curve for the formation of PC, catalyzed by CPT, which was linear for a period of 10 min. As no exogenous DAG was added it was assumed that the endogenous DAG pool was sufficient to maintain a linear activity rate for 10 min; however, it became a limiting factor as the reaction proceeded. Five minutes was defined as the reaction time for the next experiments.

The optimum temperature for CPT activity was 30ºC, as shown in figure 5. Similar values have been reported for soybean, linseed and safflower cotyledons (Harwood, 1976; Slack et al., 1985; Vogel and Browse, 1995; Vogel and Browse, 1996). The presence of more than one activity peak, a shoulder at 15ºC, suggests the existence of more than one form of the enzyme in the reaction

medium. In agreement with this hypothesis, Dewey et al. (1994) reported a small multigene family encoding CPT in soybean. The existence of enzyme forms able to act at low temperatures suggests that, at lower temperatures, they could contribute to higher production of polyunsaturated fatty acids to be used in the synthesis of structural and storage lipids. This could be one of the cold tolerance mechanisms used by the soybean plant.

The formation of PC catalyzed by CPT followed a standard hyperbolic curve of velocity versus substrate

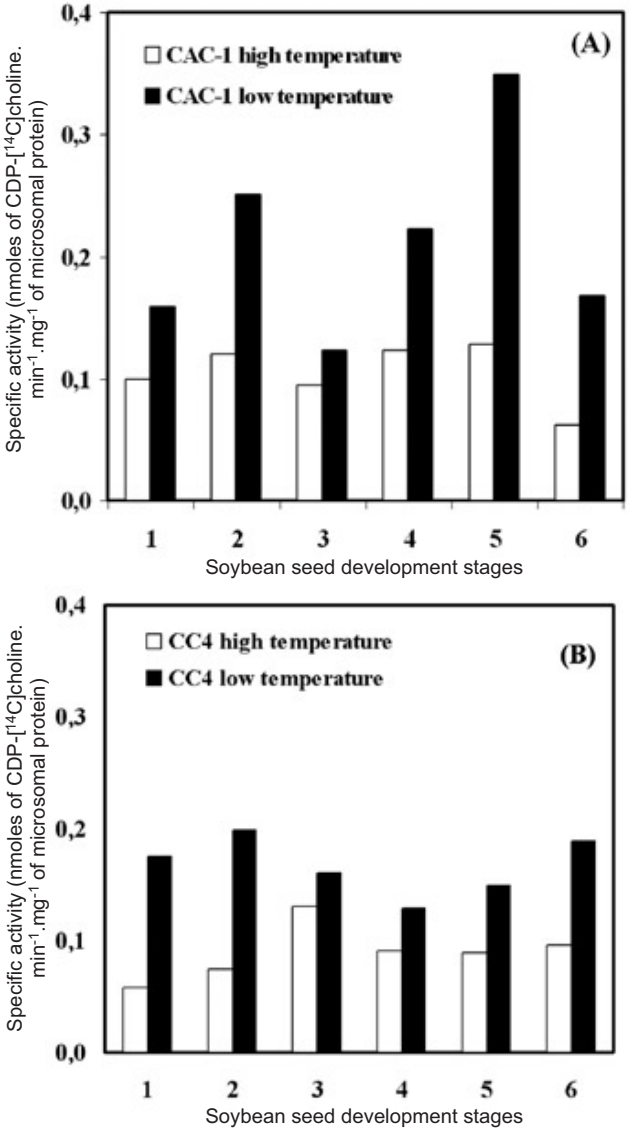

Figure 2. Cholinephosphotransferase (CPT) activities in CAC-1 (A) and CC4 line (B) soybean seeds. The plants were cultivated at 34/28ºC (high temperature) or 22/13ºC (low temperature) - day/night. Seed development stages were based on seed fresh weight: 1 - 0 to 75 mg, 2 - 76 to 150 mg, 3 - 151 to 225 mg, 4 - 226 to 300 mg, 5 - 301 to 375 mg and 6 - 376 to 450 mg. Experimental conditions: aliquots of microsomal extract corresponding to 0.1 mg of protein, Tricine-KOH buffer (pH 8.0), CDP- choline 4 x 10^{-5} mol.L^{-1} e CDP-[^{14}C]choline 4 x 10^{-7} mol.L^{-1} (0.14 MBq). The reaction mixture was incubated at 30 ºC, for 5 min.

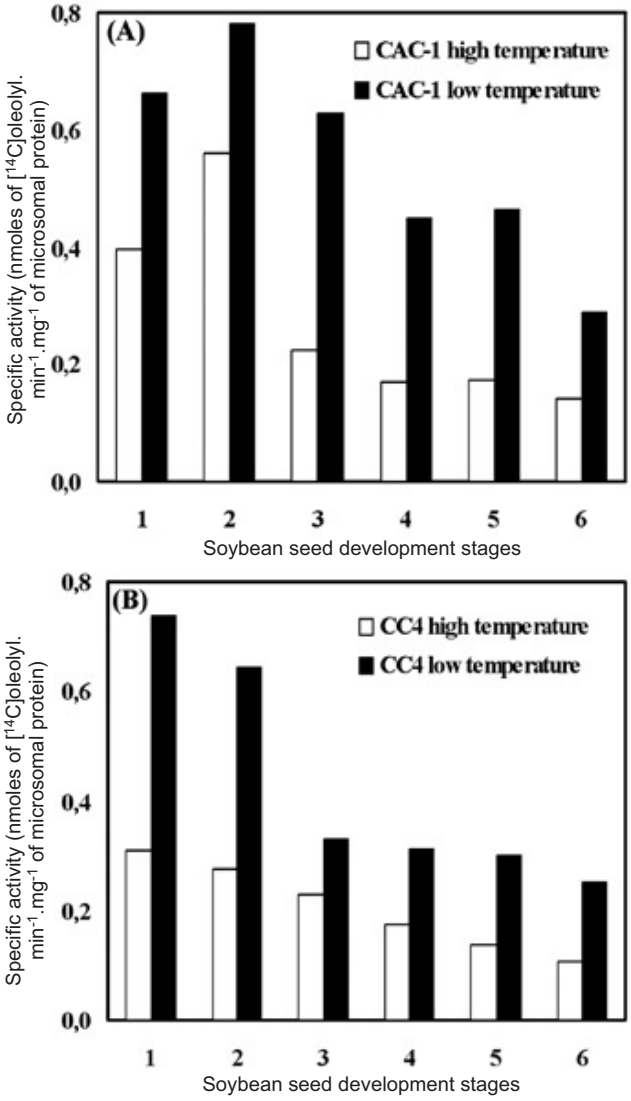

Figure 3. Lysophosphatidylcholine acyltransferase (LPCAT) activities in CAC-1 (A) and CC4 line (B) soybean seeds. The plants were cultivated at 34/28ºC (high temperature) or 22/13ºC (low temperature) - day/night. Seed development stages were based on seed fresh weight :1 - 0 to 75 mg, 2 - 76 to 150 mg, 3 - 151 to 225 mg, 4 - 226 to 300 mg, 5 - 301 to 375 mg and 6 - 376 to 450 mg. Experimental conditions: aliquots of microsomal extract corresponding to 0.12 mg of protein, potassium phosphate buffer (pH 7.2), oleoyl-CoA 1.6 x 10^{-4} mol.L^{-1} e [^{14}C]oleoyl-CoA 3.4 x 10^{-6} mol.L^{-1} (0.7 MBq). The reaction mixture was incubated at 30 ºC, for 5 min.

concentration (data not shown). The kinetic parameters obtained by non linear regression were as follows: Km_{app} of 0.23 x 10^{-5} mol.L^{-1} for CDP-choline and $Vmax_{app}$ of 89.3 nmoles of CDP-choline incorporated into diacylglycerols.min^{-1}. According to Moore (1976), the CPT from castor bean endosperm and spinach leaves have a Km_{app} of 1 x 10^{-5} mol.L^{-1} for CDP-choline; whereas that from developing safflower cotyledons has a Km_{app} of 4 x 10^{-5} mmol.L^{-1} (Slack et al., 1985). Both values differ from that obtained for soybean CPT.

The time curve for the formation of phosphatidyl-choline-oleoyl, catalyzed by soybean LPCAT, was linear throughout the analyzed period, i.e., 25 min (figure 6). A five-minute reaction time was defined for the next experiments. Griffiths et al. (1985) observed a linear rate of ^{14}C incorporation in total lipid fraction during the first 80 min for safflower LPCAT and Stymne and Stobart (1985) observed a linear activity of LPCAT from developing flax cotyledons along 40 min.

The effect of temperature on the activity of LPCAT is shown in Figure 7, in which can be observed two activity peaks for LPCAT, one between 10 and 15ºC and the other at 25ºC. The presence of more than one activity peak indicates the existence of enzyme isoforms, suggesting that some of the isoforms can contribute to increasing polyunsaturated fatty acid contents in the oil fraction, when soybeans are grown under low temperature conditions.

The formation of phosphatidylcholine-oleoyl catalyzed by LPCAT produced a standard hyperbolic curve of velocity

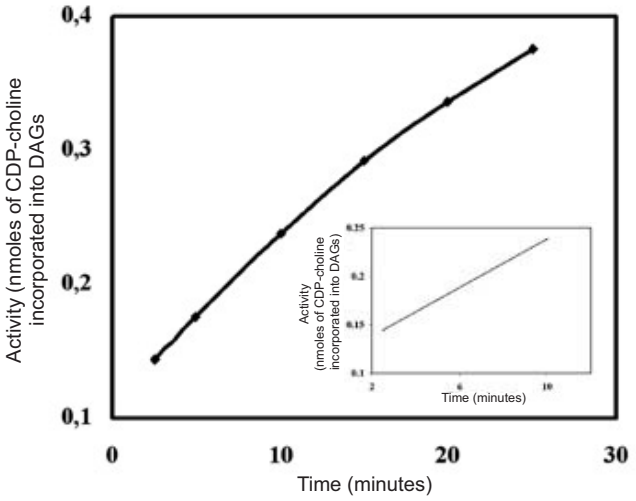

Figure 4. Time curve for the formation of phosphatidyl-choline catalyzed by CPT in soybean seeds. Experimental conditions: 0.06 mol.L^{-1} Tricine-KOH buffer (pH 8.0), 10 µL 4 x 10^{-5} mol.L^{-1} CDP-choline and 10 µL 4 x 10^{-7} mol.L^{-1} (0,14 MBq) CDP-[^{14}C]choline (tracer). Microsomal extract used, 80.5 µL. Protein concentration in the microsomal extract 1.28 mg/mL. Insert: Linear time curve for the formation of phosphatidyl-choline. The experiment was conducted at 30 ºC.

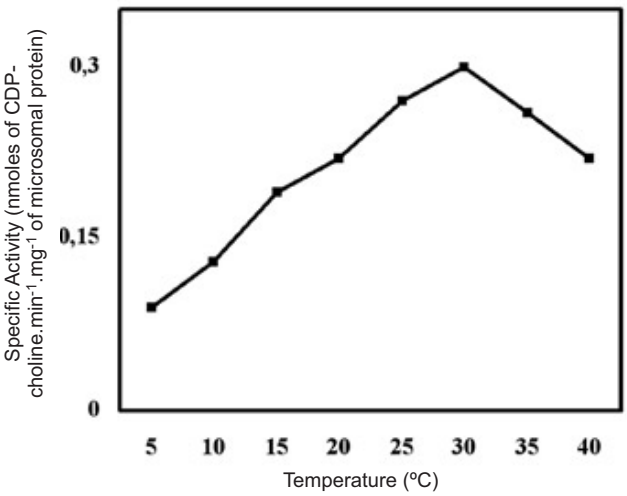

Figure 5. Effect of temperature on the formation of phosphatidyl-choline, catalyzed by CPT in soybean seeds. Experimental conditions: 0.06 mol.L^{-1} Tricine-KOH buffer (pH 8.0), 10 µL 4 x 10^{-5} mol.L^{-1} CDP-choline, 10 µL 4 x 10^{-7} mol.L^{-1} (0,14 MBq) CDP-[^{14}C]choline (tracer). Microsomal extract used, 85.1 µL. Protein concentration in microsomal extract: 1.21 mg/mL. The reaction mixture was incubated for 5 min.

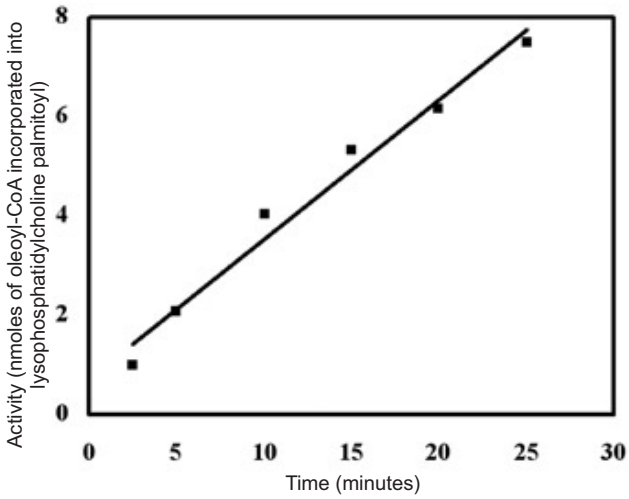

Figure 6. Time curve for the formation of phosphati-dylcholine-oleoyl catalyzed by soybean seed LPCAT. Experimental conditions: 0.1 mol.L^{-1} potassium phosphate buffer (pH 7.2), 50 µL 1.6 x 10^{-4} mol.L^{-1} oleoyl-CoA, 200 µL 3.4 x 10^{-6} mol.L^{-1} (0,7 MBq) [^{14}C]oleoyl-CoA (tracer). Amount of microsomal extract used, 77.4 µL. Protein concentration in the microsomal extract: 1.55 mg/mL. The experiment was conducted at 30 ºC.

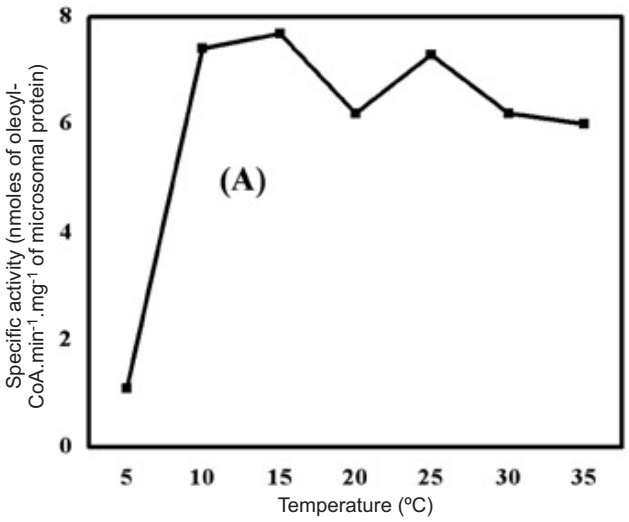

Figure 7. Effect of temperature on the formation of phosphatidylcholine-oleoyl, catalyzed by soybean seed LPCAT. Experimental conditions: 0.01 mol.L^{-1} potassium phosphate buffer (pH 7.2), 50 µL 1.6 x 10^{-4} mol.L^{-1} oleoyl-CoA; 200 µL 3.4 x 10^{-6} mol.L^{-1} (0,7 MBq) [^{14}C]oleoyl-CoA (tracer). Microsomal extract: used 92.3 µL. Protein concentration in microsomal extract: 1.30 mg/mL. The reaction was incubated for 5 min.

versus substrate concentration (data not shown). The kinetic parameters obtained by non linear regression, were as follows: Km_{app} of 0.76 x 10^{-5} mol.L^{-1} for oleoyl-CoA and $Vmax_{app}$ of 28 nmoles of oleoyl-CoA incorporated into lysophosphatidylcholine palmitoyl.min^{-1}. According to Moreau and Stumpf (1982), LPCAT from safflower has a Km_{app} for oleoyl-CoA of approximately 9.5 x 10^{-5} mol.L^{-1}.

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